BIODEGRADATION OF PETROLEUM AND ALTERNATIVE FUEL HYDROCARBONS IN MODERATE TO COLD CLIMATE

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By

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Abstract

Microbial degradation of hydrocarbon fuels contaminating soil in the Arctic and subarctic environment is a relatively slow process. Nevertheless, due to transportation and logistical limitations in rural Alaska, biodegradation might be the best and cheapest contaminant removal option.

The aim of this thesis was to investigate the environmental effects on biodegradation by naturally occurring microorganisms for some innovative hydrocarbon fuels and to determine the overall fate of hydrocarbons in soil, including degradation by fungi and bacteria, volatilization, and transport in the soil.

Three major types of fuels were investigated in small scale microcosms and larger soil columns: conventional diesel as a control substance, synthetic diesel (arctic grade Syntroleum) and different types of fish oil based biodiesel. The environmental conditions investigated included different soil types (sand and gravel), different temperatures (constant 6°C, 20°C, and fluctuating between 6 and 20°C), moisture levels (from 2% to 12% GWC), fuel concentrations (from 500 to 20,000 mg fuel/kg soil) and nutrient dosages (0 or 300 mg N/kg soil). Microbial response times and growth phases were also investigated for different inoculum types.

Conditions of 20°C, 300 mg N/kg soil, sand, $\leq$ 4000 mg of fuel/kg soil and $\geq$ 4% GWC were favorable for bioremediation, with a short lag phase lasting from one day to less

than a week, and pronounced peaks of daily CO_2 production between week 2 and 3. At suboptimal conditions, all phases were extended and slow, however at low temperatures steady metabolization continued over a longer time. The relative importance of fungal and bacterial remediation varied between fuel types. Diesel fuel degradation was mainly due to bacterial activities while fish biodiesel degradation occurred largely by mycoremediation. For Syntroleum both bacterial and fungal remediation played key roles. Volatilization contributed up to 13% to overall contaminant removal. In soil columns, degradation was slower than in microcosms, due to an uneven concentration profile of contaminants, nutrients and oxygen with depth.

In general, biodegradation showed promising results for soil remediation and the alternate fuel types were more biodegradable compared with conventional diesel fuel.

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Acknowledgements

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Preface

According to the Organization of the Petroleum Exporting Countries (OPEC) Annual Statistical Bulletin: 2008 report approximately 83 million barrels of oil per day are consumed in the world with 17 million in the United States (OPEC 2008). With this large amount being transported by land, water and/or air routes, spills of smaller or larger quantities are bound to occur. It has been estimated that 60 to 88% of total oil spills were land based, where 336 cases of between 37,800 and 375,000 liters and 74 cases with more than 375,000 liters were confirmed between 1995-1999 in the U.S. (Yoshioka and Carpenter 2002). According to the U.S. Environmental Protection Agency memorandum, between March 2008 and 2009 over 10,000 confirmed releases of petroleum hydrocarbons from underground storage tanks occurred (EPA 2009). Petroleum or non-petroleum based fuel spills result in a change of the ecosystem at different levels around the spill sites.

Hydrocarbons include toxic vapors that are directly affecting human health; however, one of the main concerns is to protect water sources from contamination.

Alternative fuels became more common during the last several decades with the aim of reducing dependency on petroleum based resources. They are generally believed to have less environmental impact. Alternative fuels in cold regions have to go through additional processing steps, which might include addition of chemicals, to achieve good performance comparable with diesel fuels. The main intention of this work was to

generate information regarding the environmental effects in moderate to cold regions on remediation of alternative non-petroleum based hydrocarbons compared with petroleum hydrocarbon.

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1 Biodegradation and Bioremediation

1.1 General Introduction and Definitions

Today's economy highly depends on hydrocarbon based fuel sources and the demand for more energy resources is increasing. Alternative fuel sources, such as vegetable or animal fat–based biodiesel, became of interest during the last few decades as a component of reducing dependency on petroleum products and to lower environmental pollution due to cleaner burning fuel. Petroleum and non-petroleum based hydrocarbons are being transported or stored in varying quantities at all times with the probabilities of unintentional spills occurring. Hydrocarbons, especially petroleum based ones, are known to be toxic to the environment and health. In general, bioremediation uses living organisms to degrade the environmental contaminants into a less toxic form. During the bioremediation process, the naturally occurring bacteria and fungi already present in the soil degrade or detoxify substances hazardous to human health and the environment (Vidali 2001). Biodegradation of individual compounds of a specific contaminant, including bioremediation of hydrocarbon polluted soils, surface- and groundwater, became of interest during the last several decades. In contaminated soil remediation systems, the top portion of the soil column is the most biologically active area, where mainly aerobic conditions exist. There are mainly two types of microorganisms present in soil remediation systems: bacterial and fungal colonies. Bioremediation is a natural waste treatment process where the microbial population increases due to growth based on the available carbon source; however, when the carbon source decreases, the microbial growth decreases as well and the remediation process slows down considerably.

The research in this thesis focuses on the environmental effects of non–petroleum based hydrocarbons in Interior Alaskan soils and the remediation effectiveness by cold–adapted indigenous microbial cultures under different conditions. The main purpose of the studies was to gather information from laboratory scale experiments that could help to make the best decision in the event of an actual field contamination.

Terms used in soil remediation systems have several definitions, which are similar, however somewhat case specific. For this thesis, the following definitions are used:

Biodegradation is the transformation of a compound through biological activity. It is the breakdown of organic contaminants (hydrocarbons) by microbial organisms into smaller compounds where the microbial organisms transform the contaminants through metabolic or enzymatic processes (Grossman and McGuire 2008).

Bioremediation takes place at contaminated sites by using technologies to enhance biodegradation processes. Bioremediation is a term where natural remedy is applied to a contaminated site cleanup process.

Mycoremediation is a term used for fungal bioremediation.

Natural attenuation of contaminants, also referred to as intrinsic or passive remediation, or natural assimilation, occurs at contaminated sites resulting in a decrease in

concentration of the contaminants over time. The term intrinsic bioremediation is used to refer to the component of natural attenuation by biological degradation mechanisms. Natural attenuation processes include physical, chemical, or biological processes that reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil and groundwater, without human involvement in the process, which is more acceptable for the public (Vidali 2001).

Aerobic degradation means that the degradation of a contaminant occurs in the presence of oxygen. Aerobic microbes are using oxygen from the air as an electron acceptor to oxidize the substrate (contaminant), and respire CO_2 as a product. Carbon of the contaminant source, among with nitrogen and phosphorous, is used to build new microbial cells. To relate carbon dioxide production quantitatively to the degradation of hydrocarbons, the following stoichiometric equation for CO_2 production was used, assuming a generalized C:H ratio of CH_2 for diesel:

$$2\ CH_2 + 3\ O_2 \rightarrow 2\ CO_2 + 2\ H_2O \qquad \text{eq. 1.1a}$$

or with a known C:H ratio for Syntroleum (Bergin 2004):

$$CH_{2.12} + 1.53\ O_2 \rightarrow CO_2 + 1.06\ H_2O. \qquad \text{eq. 1.1b}$$

During soil remediation the main degradation of the contaminant occurs under aerobic conditions, especially the upper portion of the vadose zone where oxygen supply is not limited. In general, most soil matrices contain within their pore space a sufficient amount of oxygen, which is reduced during bioremediation if the oxygen is not supplied at the rate it is used up by the microorganisms, in which case a reduction in aerobic microbial activity and consequently the rate of contaminant degradation will be observable.

1.2 Fate and transport of contaminants in the subsurface

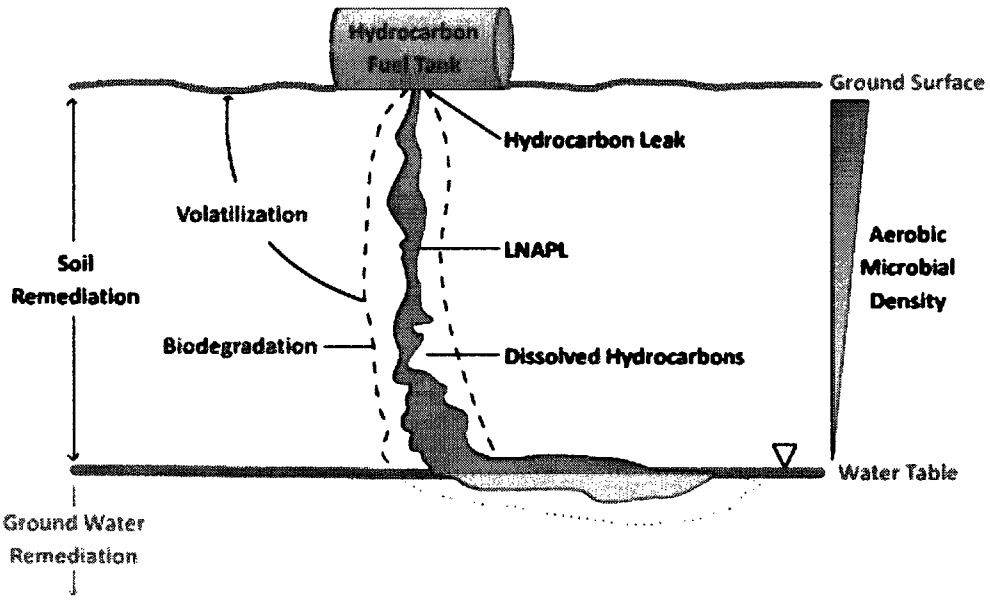

Figure 1.1 Schematic of a hydrocarbon spill.

The general schematic of a hydrocarbon spill is shown in Figure 1. While the bulk non aqueous phase liquid (NAPL) or the hydrocarbon is moving downward toward the water table, part of the fuel will volatilize and migrate through the soil pores into the air above

the ground surface, while part of the compounds will be trapped in the pore space (Figure 2). There are two types of NAPL, the light non aqueous phase liquid (LNAPL) or the dense non aqueous phase liquid (DNAPL). In this thesis all hydrocarbon fuels are LNAPLs as their density is less than water density. In close proximity of the bulk fluid, some dissolved hydrocarbons will be present, which will provide the carbon source for the microorganisms in the soil and the mainly aerobic degradation of the contaminant will take place.

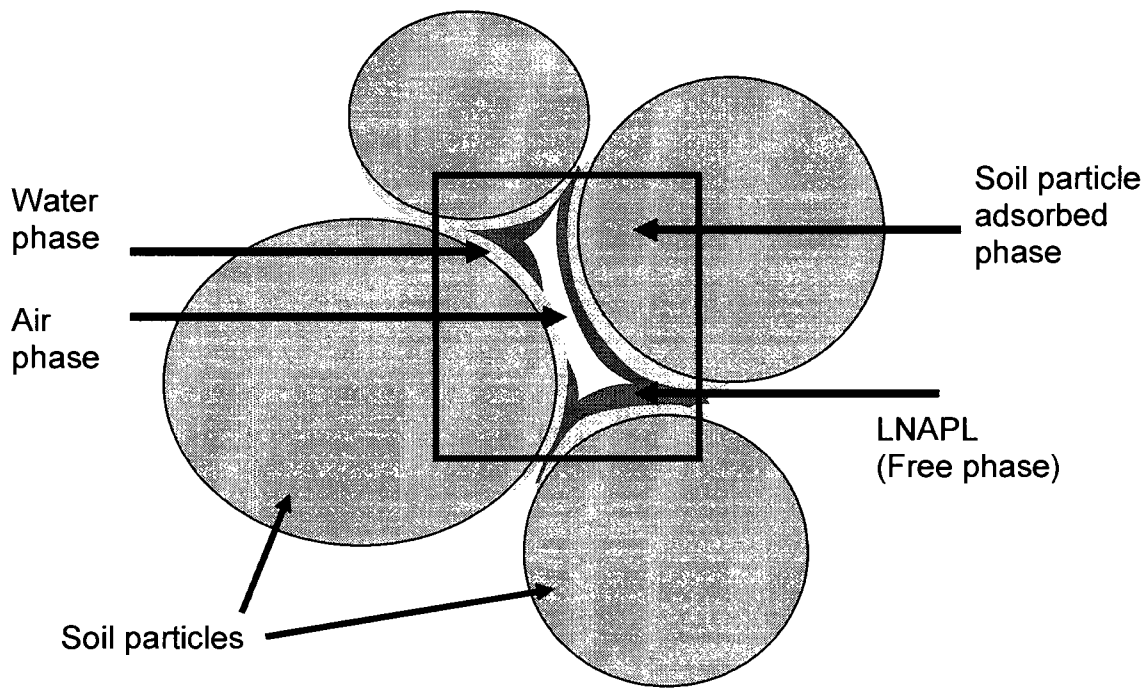

Figure 1.2 Hydrocarbon (LNAPL) distribution in soil.

In hydrocarbon contaminated soils, the hydrocarbon fraction may be present in the pore space as adsorbed fraction to the solid particles (solid phase), dissolved in soil moisture (water phase), vapor of volatile compounds (air phase) or bulk NAPL (free phase). For

low hydrocarbon concentrations, the free phase might be negligible; however as the amount of hydrocarbons present in the soil increases, the NAPL free phase will form between the water and air layers (Figure 2). Further concentration increase might result in the displacement of the water phase and later the air phase from the matrix (ME 1999). Subsurface soil particles hold water from residual water content to full saturation in a wide range. Smaller soil particles e.g. sand have a higher residual water content compared with larger soil particles e.g. gravel. The fate of hydrocarbons highly depends on the soil water content, where residual saturation can account for a large fraction of the contaminant (Selker, Keller et al. 1999).

Some parameters affecting contaminant migration:

1.2.1 Soil parameters

Subsurface heterogeneity creates significant challenges to understand migration processes through the soil and also the groundwater flow and transport. Since the majority of subsurface geology is not homogenous, but rather varies both vertically and horizontally within the subsurface, careful preparation and method development is required to accurately evaluate hydrogeologic properties. However, for simplification purposes homogeneous soil can be used during laboratory setups, which can enable some basic understanding toward the overall picture.

1.2.2 Biogeochemical processes

1.2.2.1 Kinetics

To better assess the biodegradation effectiveness and predict the time period required to achieve satisfactory contaminant removal, modeling of degradation rates is necessary. Modeling of biodegradation is an important part of any microbial remediation process. Two main types of kinetic models have been used extensively in bioremediation studies, the first–order kinetics and the Monod kinetics. The carbon mineralization data can often be fitted well by one of these models; however several studies investigated some less used kinetic models, such as parallel first-order kinetics (also called double exponential model), parallel first– and zero–order kinetic model or second-order kinetics (Sleutel, De Neve et al. 2005).

First–order degradation kinetics follows an exponential decay expression, based on the assumption that the degradation rate is proportional to the remaining substrate, i.e. fuel concentration. Rate constants can be determined by using the integral method based on the calculated amount of substrate still available. The first–order kinetics fit well for most biodegradation data; it is a representative model for hydrocarbon mineralization (Aichberger, Hasinger et al. 2005). The following equation represents first order kinetics

$$\ln C_t = \ln C_0 - kt \qquad \text{eq. 1.1}$$

where C_t is the remaining contaminant concentration at any given time t (based on respiration data); C_0 is the initial contaminant concentration; and k is the rate constant. According to this equation, in a plot of ln C_t versus time, the data points should fall on a straight line with the slope $-k$ if first–order kinetics apply, that is, if the rate of degradation is proportional to the remaining amount of contaminant at any given time. The rate constant can be easily obtained from the slope of a regression line. The first–order rate kinetics are used very often in microbial degradation studies (Tian, Kang et al. 1992; Sleutel, De Neve et al. 2005).

Monod kinetics offers insight into biodegradation mechanics because they address biomass growth as well as a nonlinear saturation–type dependence of the rate on substrate concentration (Ostendorf, Schoenberg et al. 2007). The Monod equation is used by many degradation studies (Monod 1949) to describe experimental data where the microbial growth rate is dependent on the substrate concentration. The Monod equation can be written as:

$$\mu = \frac{\mu_{\max} \; S}{K_S + S} \qquad \text{eq. 1.2}$$

with μ the specific growth rate, μ_{max} the maximum specific growth rate, S the substrate concentration, and K_S the half saturation constant.

1.2.2.2 Microbial growth phases

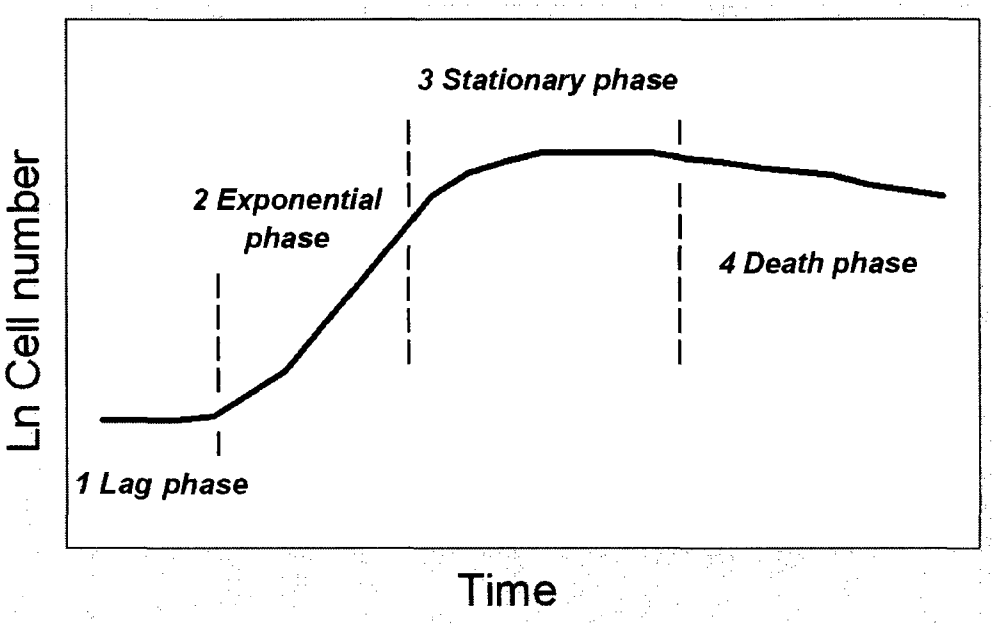

Figure 1.3 Typical microbial growth curve.

The schematic Figure 3 shows the general microbial growth phases, where the first phase (1) represents the lag phase, the second (2) the exponential growth phase, the third (3) the stationary phase and the fourth (4) represents the death phase.

Lag phase

During the lag phase, the microbial cultures are adapting to the new substrate as an energy source (hydrocarbon) and the cell growth is very slow or minimal. This may take from few hours up to several weeks. During this time, vertical migration of the contaminant occurs without significant depletion of the overall contaminant quantity due to microbial activities. The deeper the contaminant moves into the vadose zone, the less

oxygen is present for the aerobic degradation. Depletion of oxygen can inhibit degradation. The shorter the lag phase, the more effective the bioremediation process and for this reason it is important to shorten this phase and ensure long exponential and stationary phases when the microbial activities are highest.

Exponential phase

During this microbial growth phase, there is a significant increase in microbial activity. In a typical batch bacterial growth curve, the lag phase is followed by exponential growth and later a declining rate of growth when substrate availability is reduced; however the cell density is still somewhat increasing. These growth phases are ultimately followed by a stationary and death phase where microbial numbers decline (Vaccari, Strom et al. 2006). In the exponential phase the degradation rate is the highest, which means that the longer the exponential (and stationary) phase the faster and more complete the substrate consumption.

Stationary phase

During the stationary phase, microbial respiration is still significant and microbial numbers are high; however the microbial population is steady or declining and substrate utilization rates decrease considerably. The substrate utilization for maintenance might also be added to the microbial growth kinetic equations, which, consequently, might increase substrate consumption. The stationary phase may be entered when the substrate concentration is limiting, which in the case of hydrocarbon spills is the remediation goal;

however this phase also can be due to toxin accumulation, which than also causes entering the death phase.

Death phase

During the death phase, negative growth or death can occur due to substrate limitations, toxins, predators (other microbes) and physical factors (e.g. shear stress). These phases are generalized phases assuming environmental parameters remain unchanged; however when more nutrients are added to the soil, an increase in temperature occurs etc., a secondary exponential phase can be achieved and ultimately the mineralization of the substrate can become more complete.

1.3 Environmental parameters affecting bioremediation

1.3.1 Naturally occurring microorganisms

For hydrocarbon contamination in Alaskan lands, in situ remediation can be one of the most feasible processes under given circumstances. When fuel is spilled in rural areas, natural biodegradation might be the best and cheapest removal option, especially in the Arctic and subarctic environment, where the limited infrastructure and difficulties in transportation can be limiting factors for a fast response. Contaminant removal by indigenous microbial species already present in the soil requires minimal or no human involvement and also can achieve a positive outcome on overall contaminant removal. The presence of cold-adapted hydrocarbon degraders in the soil was confirmed by several earlier studies which found that significant numbers were present (Whyte, Greer et al.

1996; Aislabie, Foght et al. 2000; Bej, Saul et al. 2000). Addition of cultivated bacterial cultures might have significantly lower degradation effectiveness compared to laboratory results when applied in Alaskan soil, where the microorganisms compete for the substrate with indigenous species (Leahy and Colwell 1990). When laboratory studies are performed with microorganisms from Alaskan soil, the lab results can be more applicable to the field. Microbial degradation of hydrocarbons in cold environments might be underestimated in laboratory studies compared with field studies as some research suggests (Hohener, Dakhel et al. 2006).

Microbial community composition change after hydrocarbon exposure

Hydrocarbon spills result in an elevated carbon level in the subsurface, which can inhibit microbial growth due to toxic levels, or enhance microbial growth and activities (Atlas and Bartha 1998). Previous exposure to hydrocarbons has an important effect on the microbial communities' ability to degrade the substrate in the environment at a higher rate, compared with no prior exposure (Leahy and Colwell 1990). For remediation purposes the shorter the lag phase, the more complete mineralization might be achievable for a given time period. It would be advantageous if the lag phase can be eliminated or extensively shortened by using indigenous microorganisms already adapted to the specific fuel type.

1.3.2 Environmental factors

It is known that microbes need nutrients, carbon, and energy to survive and multiply. Biodegradation rates are, therefore, highly dependent on surrounding environmental conditions such as temperature, nitrogen availability and oxygen supply, whose impact was investigated in this thesis. It is important to know the influence of the physical and environmental factors to predict the effectiveness of naturally occurring microbial activities for soil remediation. When a fuel spill occurs, the first few weeks, where the adaptation of the microbes to the new substrate takes place, are crucial in the remediation progress. The faster the microorganisms in the soil adapt to the changed environment, the more complete the degradation of the substance in a limited time frame. Several laboratory tests have shown relatively high total hydrocarbon degradation during a short period of time such as 27% of diesel degradation after one day in an aquatic environment (Zhang, Peterson et al. 1998) and up to 80% during 38 days in the terrestrial subsurface in cold climates (Margesin, Hammerle et al. 2007), while some field studies showed much less degradation over a longer time period, even after years (up to 68% during 447 days) (Margesin and Schinner 2001). Good correlations between laboratory and field studies have been also reported; however, diesel contamination tends to persist in the environment more than in the laboratory (Zytner, Salb et al. 2001).

The following factors were investigated in the thesis: air temperature, soil moisture levels, soil types, nutrient levels and microbial inoculation.

1.3.2.1 Temperature

Biodegradation of hydrocarbon fuels by indigenous microorganisms in the Arctic and subarctic environment is a relatively slow process due to long term winters and brief periods of relatively high temperatures during summer seasons. For microbial cultures in an arctic and subarctic environment, a temperature range of 1°C to 25°C can be assumed normal based on general summer air temperatures of Interior-Alaska. The respiration rates and O_2 consumption of cold-adapted microorganisms increase with rising temperature (Atlas and Bartha 1998; Ferguson, Franzmann et al. 2003b; Walworth, Woolard et al. 2003). The correlation between temperature and degradation rates has been extensively studied for petroleum based hydrocarbons where minimal or no mineralization of the contaminant occurs when the temperature drops near or below freezing conditions (Ferguson, Franzmann et al. 2003b).

The relationship between different temperatures and reaction rate constants is commonly described by the Arrhenius equation (1.3). The Arrhenius equation can be applied to biological systems, assuming that biological behavior and chemical reactions are affected by temperature similarly. Chemical reactions and biological mineralization increase with increasing temperature,

$$k = A_f \exp\left(\frac{-E_a}{RT}\right)$$

or rearranged:

$$\ln k = \left(\frac{-E_a}{RT}\right) + \ln\left(A_f\right) \qquad \text{eq. 1.3}$$

where k is the temperature dependant rate constant; A_f is the pre-exponential factor determined from the Arrhenius plot; E_a is the activation energy also determined from the Arrhenius plot; R is the gas constant (8.314 J/mol); and T is the temperature in Kelvin.

To determine the characteristic times it takes the soil to correspond to the air temperature, the following equation can be used:

$$CT \approx \frac{L^2}{\alpha} \qquad \text{eq. 1.4}$$

where CT represents the characteristic time (seconds), L is the length approximated as half of the soil column, which is the vertical distance from the top of soil surface, and α is the thermal diffusivity (m^2/s) and calculated from

$$\alpha = \frac{k}{(\rho * c_p)} \qquad \text{eq. 1.5}$$

where k represents the thermal conductivity of the soil (W/mK), ρ is the density of the soil (kg/m^3) and c_p is the specific heat (J/kg K).

To see if the soil temperature at different depths can be modeled as a function of the air temperature, the following equation can be used (Cengel 2007) with the assumption of heat flux is being constant:

$$\frac{T(x,t) - T_{air}}{T_{soil} - T_{air}} = erfc\left(\frac{x}{2\sqrt{\alpha t}}\right) \qquad \text{eq. 1.6}$$

where α is the thermal diffusivity (m^2/sec), t is time (s), x is the distance (m) of the thermocouple placed in the soil from soil surface varying between 0 to L, T_{air} is the air temperature (K), and T_{soil} is soil temperature (K).

1.3.2.2 Nutrients in soil

The soil nutrient concentration is a major factor that influences the degradation process. Too low or too high nitrogen in the soil can inhibit degradation processes (Ferguson, Franzmann et al. 2003a; Walworth, Woolard et al. 2003). The collected Interior-Alaskan soil samples showed initially minimal available nutrient content, which has been noted in other cold region studies as well (Walworth, Pond et al. 2007). Addition of nutrients to

enhance microbial degradation process in cold climate is very often necessary (Margesin and Schinner 1997; Margesin and Schinner 2001; Schiewer and Niemeyer 2006), and it is a relatively cheap option within regulatory limits.

According to the mass energy balance theory (for aerobic chemo-organotrophic microbial growth), the use of substrate and nitrogen in the presence of oxygen will produce new biomass plus a product (Panikov 1995),

$$CH_mO_l + a\ NH_3 + b\ O_2 = Y_c\ CH_pO_nN_q + Y_p\ CH_rO_sN_t + (1 - Y_c - Y_p)\ CO_2 + c\ H_2O$$

Substrate biomass product

According to the above statement, the yield of microbial biomass per organic substrates indicates how much carbon and nitrogen is converted to microbial cells. In biodegradation studies the total carbon mass balance might not be closed if some carbon is not directly accounted for e.g. due to volatilization.

1.3.2.3 Soil particle size

The particle size of the soil influences the amount of microbes present in the soil. For example sand particles are relatively large grain sized soil particles with relatively small surface area and consequently have relatively few attached microbes compared with clay particles, which hold more moisture and also organic matter (Lynch and Hobbie 1988).

1.4 Fuel Types

1.4.1 Conventional diesel and diesel heating fuels

Diesel fuel degradation in cold climate is well documented and literature on this topic is readily available. Therefore, when investigating alternative fuel degradation diesel fuel is a suitable control substance.

1.4.2 Syntroleum

Arctic grade Syntroleum is produced from natural gas and processed through the Fischer-Tropsch gas-to-liquid technique by the Syntroleum Corporation. Syntroleum can be used in its pure form or as a blend with conventional diesel fuel without any mechanical modification to an existing diesel engine (FTA 2007). Since Syntroleum use in cold climates is still in the pilot phase, its environmental effects and biodegradability are less known. However Syntroleum is expected to be more degradable compared with conventional diesel fuel due to its higher content of straight aliphatics (C_{8-28}), which are typically more easily degradable than branched or cyclic hydrocarbons occurring in conventional diesel fuel.

1.4.3 Fish oil and fish biodiesel

Biodiesel is a source of renewable energy that can easily be stored and is not as affected by variable weather as solar and wind energy. Biodiesel is compared with diesel fuel a more environmentally friendly non-petroleum based hydrocarbon fuel made from renewable sources: fish/animal fat. The proposed application of the fish biodiesel is for

use as a heating fuel in rural Alaskan communities, mixed with high sulfur diesel fuel. Biodiesel in its pure form is rarely used in existing engines; instead different percentages of blends are more commonly used.

Prior to 2004, Alaska generated 8 million gallons of fish oil per year as a byproduct of the Alaskan fish industry (Steigers, Seshadri et al. 2004), which increased up to 13 million gallons by 2007 (AEA 2007). Collection of the raw fish oil is being organized from different parts of Alaska, from where it could be transported in large qualities from 2009 onwards (AEA 2007). The fish oil biodiesel used here were comprised of biodiesel that had been degraded by oxidation and oxidized biodiesel that had been rehabilitated. The biodiesel used in the biodiesel blend experiments came from rehabilitated biodiesel stock, while other chapters report data for both biodiesel types. After the biodiesel was shipped back to Alaska, some oxidation had already occurred. To reduce oxidation, 400 ppm by volume of the antioxidant ethoxyquin was added. After the addition of the antioxidant, the biodiesel was passed through a bleaching column of clay (rehabilitated) to remove fats and oxidized components that might still present (Schmid, 2009, Pers. Comm.). The purpose of the treatment was to enable good engine performance and also lower the risk of engine failure. The effects of the fish oil and fish biodiesel on the environment (e.g. on soil permeability) and its fate needed to be investigated due to the unknown consequences in the extreme surroundings.

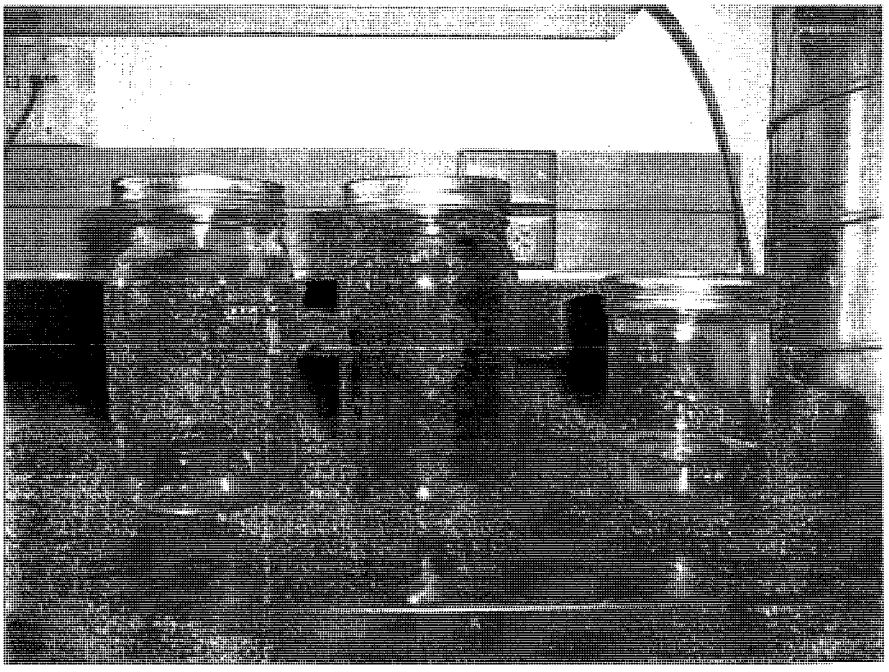

Figure 1.4 Syntroleum (left), B100 – Toklat fish biodiesel (FBD1) (right) and phase two fish biodiesel (FBD2).

1.5 Undertaken research

Small and larger scale studies of biodegradation experiments were carried out under varying physical and environmental conditions explained in more detail in earlier paragraphs of this chapter. Schematics of the experimental setups are shown in Figures 5 and 6. The majority of the literature available on hydrocarbon bioremediation involves petroleum based products, therefore in this research newly developed alternative fuels were investigated with limited to no literature available regarding their degradability in cold climate.

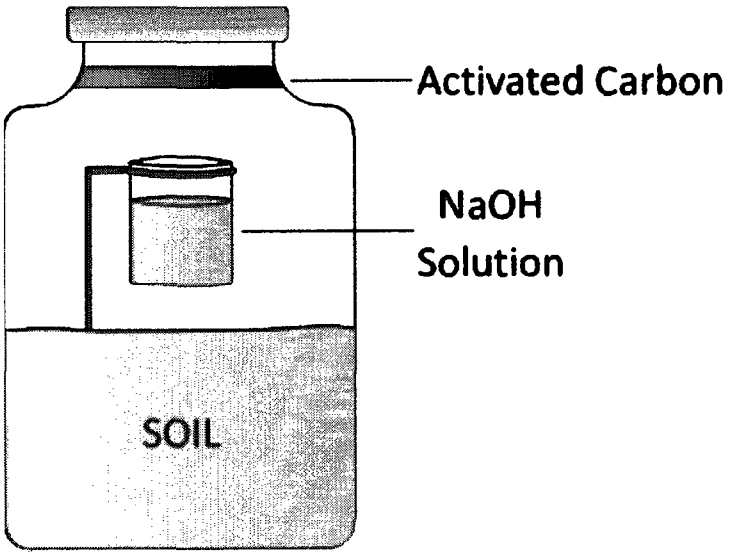

Figure 1.5 Schematic for small scale experiments.

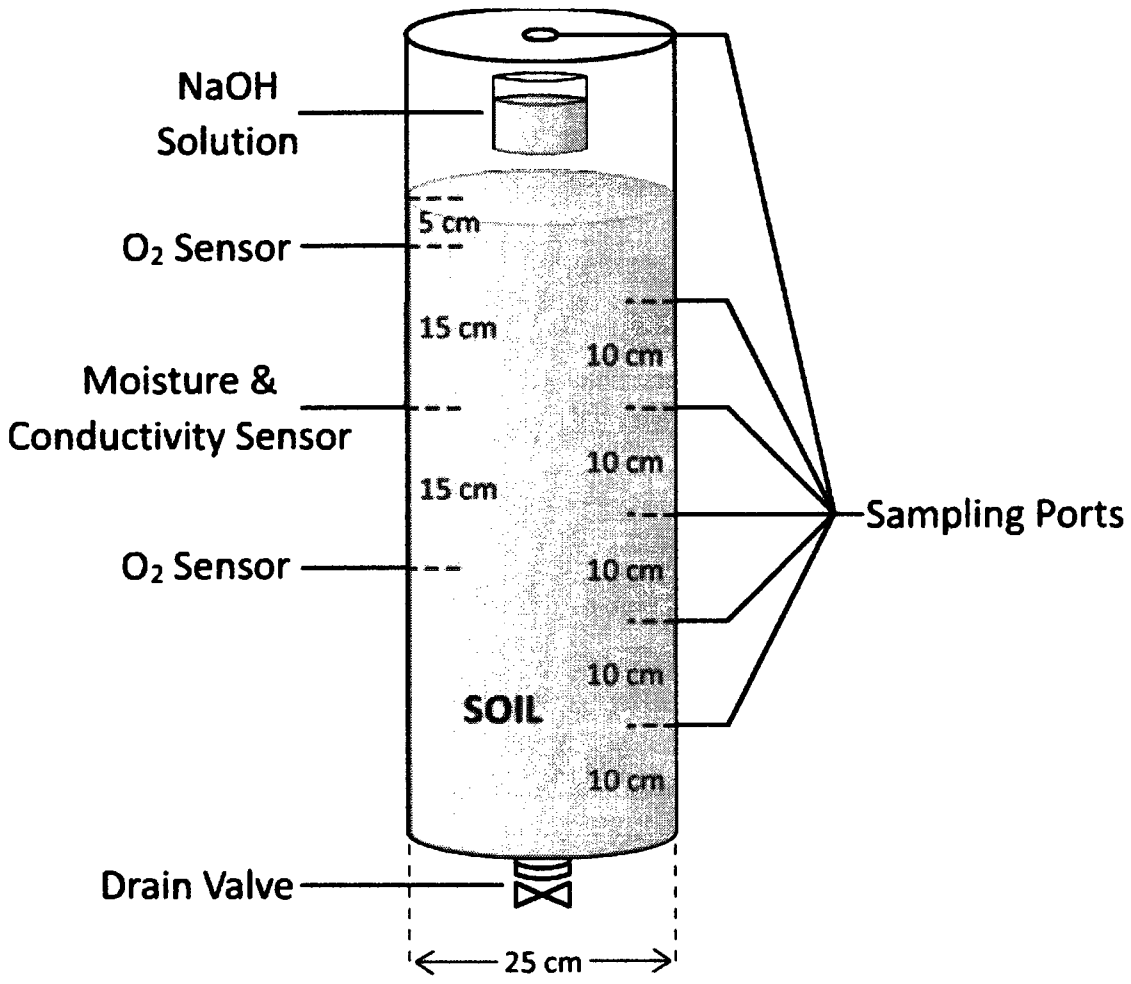

Figure 1.6 Schematic for large column setup.

All of the papers in this thesis include microbial respiration measurements as one of the easiest methods to implement, it is somewhat labor intensive but relatively inexpensive and the produces good data.

Chapters 2 to 4 are based on similar experimental setups while varying environmental parameters and fuel types. The main methods involved most probable number (MPN) (de Man 1983; Haines, Wrenn et al. 1996), gas chromatography – mass spectrometry

(GC/MS) for diesel range organics, high performance liquid chromatography (HPLC), general ASTM standards and elemental combustion system for general soil analysis. These methods provided information regarding soil hydrocarbon, nutrient and microbial population density.

Chapter 5 contains activated carbon involvement in the experimental setup and its overall effects were examined with gas chromatography – mass spectrometry (GC/MS) to determine volatiles. Chapter 4 also used DNA sequencing to identify the fungal colonies mainly responsible for mycoremediation processes (White, Bruns et al. 1990; April, Foght et al. 2000).

Chapter 6 focuses on larger scale experimental setups and includes also computer simulation of the fate of different compounds.

Hypotheses

1) In contaminated soils several environmental factors can enhance biodegradation rates. Higher temperature, lower hydraulic conductivity, sufficient moisture and nutrient content increases petroleum hydrocarbon contaminant removal by microbial activities in soil and is therefore also expected to increase mineralization of synthetic diesel and fish biodiesel fuels. To test this hypothesis several batch experiments were set up in the laboratory.

2) Mass transfer limitations (for oxygen and nutrients) can have a negative effect on degradation rates as oxygen is quickly used up during aerobic processes and replaced with CO_2, and non–homogeneous nutrient distribution results in too low or too high nutrient concentration, which inhibits contaminant removal efficiencies for petroleum or non-petroleum based hydrocarbons by microbial activities. For this purpose several batch experiments were conducted where daily or bi-daily agitation was applied also larger-scale column experiments were implemented.

3) If larger amounts of fuel are spilled, transport of fuel in the soil will lower the amount biodegraded since oxygen is not readily available deeper in the soil as a free phase or bulk contaminant replaces air phase in the soil pores, leaving less available oxygen present for aerobic degradation of the synthetic diesel and fish biodiesel.

4) Addition of natural microbial cultures from Alaskan soils contaminated with a specific fuel enhances biodegradation effectiveness for that fuel type and considerably shortens the adjusting times to the substrate (lag phase).

1.6 Brief overview of the papers embedded in this thesis as Chapters 2 to 6

Chapter 2 shows the results for long term degradation of the hydrocarbon contaminated soils samples while varying environmental conditions, such as temperature, moisture content, nutrient dosages and soil types. The paper was based on the biodegradation of two types of fuels, conventional diesel and arctic grade Syntroleum by naturally

occurring microbes. This chapter identifies optimal conditions for biodegradation of hydrocarbon fuels.

Chapter 3 shows degradation of a third fuel type, raw fish oil, compared with Syntroleum and conventional diesel fuel. This chapter also investigated the microbial growth phases and the effects of previously adapted microbial inoculum to a specific fuel type. Cultivated microbial inocula were added to contaminated soils to investigate adaptation times for remediation purposes.

Chapter 4 investigates fish biodiesel a step further, when added to heating diesel. Several fish biodiesel blends were prepared and their biodegradation in soil was studied.

Chapter 5 focuses on fungal remediation and survivability under different conditions. Chapter 5 also includes activated carbon use in the experimental setup and its adsorptive removal of volatile compounds from the headspace, including the effect of the reduced volatile hydrocarbon amount on degradation rates.

Chapter 6 focuses on a larger scale experimental setup compared to small scale experiments both under optimal conditions. Computer modeling of biodegradation ad transport of alternative fuels was also performed by using HYDRUS 2D/3D software.

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2 Investigation of the physical and chemical parameters affecting biodegradation of diesel and synthetic diesel fuel contaminating Alaskan soils[1]

2.1 ABSTRACT

In cold regions, biodegradation of fuel spills can take a prolonged period of time. Conventional fuels and crude oil contain contaminants such as aromatics and PAH which can pose risks to humans and the environment. The goal of the present study was therefore to investigate the biological degradation of an alternative synthetic fuel, Syntroleum, which is less toxic and, as shown in this study, more easily biodegradable than conventional diesel fuel. Use of alternative fuels such as Syntroleum would be especially beneficial in sensitive regions where spills of conventional fuel are highly undesirable. Gravel and sand from Interior Alaska was spiked with diesel and synthetic diesel fuel (arctic grade Syntroleum). After adding an inoculum, samples were incubated in the laboratory at different temperatures (6°C and 20°C), contamination levels (2000 mg and 4000 mg of fuel/kg dry soil), nutrient dosages (300 mg N/kg soil and 0 mg N/kg soil) and moisture contents (2%, 4%, 8% and 12% gravimetric water content). The

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objective of this research was to investigate the effect of physical and chemical environmental conditions on the biodegradability of contaminants and to determine optimal conditions for biodegradation by indigenous microorganisms. The respiration rate (CO_2 production) was measured as an indicator of microbial activity and mineralization of contaminants, and complemented by analysis for hydrocarbons at the end of the experiment by gas chromatography/mass spectrometry. Both fuel types were biodegraded, with up to 75% mineralization after 17 weeks. The faster degradation rate was achieved in Syntroleum contaminated soils with a degradation rate constant of 0.0064–0.0106 d^{-1} at 20°C. At 6°C, diesel fuel showed minimal degradation during several short-term studies (4–6 weeks), less than 5% total mineralization of the hydrocarbons in the fuel. The average degradation-rate constant for Syntroleum at 6°C was 0.0016 d^{-1} during a 4-week study, while the degradation rate constants became much higher (0.0045–0.005 d^{-1}) for the long-term experiments (12–17 weeks), resulting in significant mineralization of total carbon present. The different moisture contents in the sandy soil showed no significant impact on respiration. The addition of fertilizer was essential to achieve good degradation rates. After the end of a the 17–week experiment, the recovered contaminant was approximately 50% less in the case of Syntroleum when nutrients were added to the soil as compared with nutrient-deficient conditions. Respiration rates were higher in sand than in gravel, which may be due to differences in soil porosity and the available surface area for more even hydrocarbon distribution. Degradation rates varied significantly over time. A first–order model, which used different rate constants for three growth phases, was able to model cumulative carbon

dioxide production quite well over a period of four months. In the carbon mass balance, the sum of the diesel range organics recovered from the soil plus the produced carbon dioxide accounted for approximately 30–85%. The remaining amount of carbon either was incorporated into biomass, degraded incompletely, or evaporated.

2.2 INTRODUCTION

Alternative fuel sources are becoming more important in today's economy. For hydrocarbon contamination in Alaskan lands, in situ remediation can be one of the most feasible processes under certain circumstances. When fuel is spilled in rural areas, natural biodegradation might be the best and cheapest removal option. Addition of cultivated bacterial cultures might have significantly lower degradation effectiveness compared to laboratory results when applied in Alaskan soil, where the microorganisms compete for the substrate with indigenous species (Leahy and Colwell, 1990). When bacterial culture is cultivated with microorganisms from Alaskan soil, the lab results can be more applicable to the field. Microbial degradation of hydrocarbons in laboratory studies in cold environments might be underestimated compared with field studies as some research suggests (Höhener et al., 2006). It is important to know the influence of the physical and environmental factors to predict the effectiveness of naturally occurring microbial activities for soil remediation.

It is known that microbes need nutrients, carbon, and energy to survive and multiply. Biodegradation rates are, therefore, highly dependent on surrounding environmental

conditions such as temperature and moisture supply, whose impact was investigated in this study. This study investigated the effect of temperature (6° C vs. 20° C), moisture content (2–12% gravimetric water content), contamination level, mixing, and nutrient addition (0 vs. 300 mg N/kg soil) on the biodegradation of two fuel types by naturally occurring microbes in two types of soil (sand and gravel) in microcosm experiments. Biodegradation was characterized by measuring CO_2 production during the course of the experiment and by GC/MS analysis of hydrocarbons remaining at the end of the experiment. Conventional diesel fuel was compared with Syntroleum fuel, a synthetic fuel derived from natural gas.

Syntroleum is a synthetic diesel fuel processed through the Fischer-Tropsch gas-to-liquid technique. Syntroleum is produced from domestically available feedstocks, such as natural gas or coal. The advantages of Syntroleum are that it is less toxic than conventional diesel due to its minimal content of aromatics and that it is a low-emission fuel that does not require changes to vehicle engines or infrastructure (FTA, 2007). During storage and transportation of the fuel, spills may occur. Since Syntroleum is still in the developmental and test phase regarding cold climate performance, its environmental effects and biodegradability are unknown. Syntroleum has a higher content of straight aliphatics, which are typically more easily degradable than branched or cyclic hydrocarbons occurring in conventional diesel fuel. To prove the hypothesis that Syntroleum can be degraded faster than diesel, research such as that conducted in this study is necessary.

2.3 MATERIALS AND METHODS

2.3.1 Methods

The rate of hydrocarbon degradation was studied as a function of time while varying the fuel type, contaminant dosage, temperature, moisture content, nutrient dosage, and soil types.

For each experiment, 1 kg of soil (sand or gravel) was placed in an airtight 2.5 L container. Quantified amounts (2000 mg/kg or 4000 mg/kg dry $soil^{-1}$) of the chosen contaminant, arctic-grade Syntroleum or conventional diesel (#2), were added to the surface of previously uncontaminated soil. Additionally, a small amount of previously contaminated soil from an Interior Alaskan automobile reclamation center was added to provide an inoculum of microbes. Fertilizer of the type 20-20-20 ($N\text{-}P_2O_5\text{-}K_2O$, where the total nitrogen ingredients were: 20% Ammonia N, 30% Nitrate N, and 50% Urea nitrogen) was dissolved in 10 ml of water with a final concentration of 30 mg N/ml of solution, and was sprinkled over the soil surface, achieving nutrient dosages of 0 mg N/kg vs. 300 mg N/kg dry soil. Adjustments were made to attain the same water content of the soil with or without additional nutrients. Except when soil agitation was specifically investigated, the soil was not mixed after or during these additions to better represent natural conditions after a spill on the soil surface. The samples were incubated either at 6°C or 20°C. For most experiments, some water was added, maintaining a gravimetric moisture content fluctuating between 4 and 6%. In one set of experiments,

moisture content of 2%, 4%, 8%, and 12% were deliberately adjusted in different containers.

Data were collected over different time periods, ranging from 4 weeks to 17.5 weeks. Carbon dioxide is the main product generated when a substrate is completely mineralized in aerobic degradation processes. Therefore, the respiration rate, that is, the rate of CO_2 production, was measured as an indicator for microbial activity. Typically, the container was opened once daily for re-aeration and to measure the evolved CO_2, which had been captured in 20 ml of 1N NaOH solution (Page et al, 1982). During the daily analysis of the small-scale experiment, CO_2 measurements were taken by using a method and formula developed by Stotzky (Anderson, 1982). The carbonate formed was precipitated with a 0.3N $BaCl_2$ solution.

To relate carbon dioxide production quantitatively to the degradation of hydrocarbons, the following stoichiometric equation for CO_2 production was used, assuming a C:H ratio of $CH_{2.12}$ for Syntroleum (Bergin, 2004):

$$CH_{2.12} + 1.53\ O_2 \rightarrow CO_2 + 1.06\ H_2O. \qquad \text{eq. 2.1}$$

This stoichiometric equation applies only to mineralization of the fuel. The amount of CO_2 measured as a result of microbial consumption of hydrocarbons might be different from the actual hydrocarbon decrease in the soil. Use of this equation to estimate the amount of fuel degraded based on the amount of CO_2 produced can lead to over- or underestimations. The amount of fuel degraded may be underestimated based on CO_2

measurements, since not all substrate is completely mineralized, some being incompletely degraded or used for the production of new biomass. On the other hand, the total fuel degraded may be overestimated if some CO_2 is produced from other carbon compounds originally present in the soil. That scenario is unlikely in the present study, however, since soils with low organic content were used. The predominant importance of the added fuel compared to other possible substrates was confirmed by the use of controls without fuel addition. A baseline was developed based on uncontaminated soil samples for comparison with the calculated CO_2 production of the degradation experiments.

First-order degradation rates were determined by using the integral method based on the calculated amount of substrate still available according to respiration data,

$$\ln C_t = \ln C_0 - k\,t \qquad \text{eq. 2.2}$$

where C_t is the remaining contaminant concentration at any given time t; C_0 is the initial contaminant concentration; and k is the rate constant. According to this equation, in a plot of $\ln C_t$ versus time, the data points should fall on a straight line with the slope $-k$ if first-order kinetics apply, that is, if the rate of degradation is proportional to the remaining amount of contaminant at any given time. The rate constant can be obtained from the slope of a regression line.

To evaluate the relationship between substrate use and CO_2 production, gas chromatography/mass spectrometry (GC/MS) analysis was performed (Agilent Technologies 6890N Network GC System coupled to a 5873 mass selective detector) and

compared to respiration data. For this purpose, the diesel range organics (DRO) determination was conducted using a modified method that was developed based on the AK 102 (ADEC, 2002) and EPA 8270 (semi volatile organics by GC/MS) methods (EPA, 1996). Recovery data analysis was performed for determination of the actual hydrocarbon recovery value by the gas chromatography method used. For this purpose, a known amount of fuel was added to the soil and extracted immediately. The quantity of the average highest recovered hydrocarbon amount for each fuel type was taken as a reference value of 100% for the GC/MS data analysis instead of the theoretical value (calculated by using a generalized stoichiometric equation). This was done due to some interference with the solvent during the first few minutes of the GC/MS spectrum. The lower weight volatile compounds, in particular, are not reliably measured, since their peaks occur at a time when the solvent is still eluted.

2.3.2 Soil characteristics

The soil used in the experiments was not sterilized and thus contained some naturally occurring microbial cultures that survive extreme conditions (–50° C to 30° C). The purpose for using unsterilized soil was to further simulate natural degradation processes. General soil analysis was performed using the instrument a Dionex DX 500 ion chromatograph with PeakNet software for nitrite and nitrate analysis and the ASTM standard for sieving.

The pH of the soil was analyzed before the experiments, using a Mettler Toledo pH meter at 21.8°C. The soil pH value was between 7.07 and 7.25, which is optimal for hydrocarbon degradation (Saadoun and Al-Ghzawi, 2005). The original water content before the start of the experiments was less than 1%. The bulk density and porosity of the soil was determined by using the ASTM standard. The average bulk density was 1.6 g/cm^3 for sand and 1.79 g/cm^3 for gravel. The average soil porosity was 39.7% for sand and 32.5% for gravel.

2.4 RESULTS

2.4.1 Effect of fuel type on respiration rates

Experimental data generated over a period of four months show that the two types of fuel (Syntroleum and diesel) had similar biodegradation trends, whereby the synthetic Syntroleum generally had higher rates than diesel (Figures 1, 2, 3, 5, 6, 10, and Table 1). Figure 1 shows a linearized plot for determining overall respiration rate constants for diesel and Syntroleum that were attained in fertilized soil incubated at 20°C with different fuel dosages. At the end of the 17-week study, the Syntroleum rate constant was 65% higher compared with diesel, with constants of 0.0066 d^{-1} and 0.0041 d^{-1}, respectively. A comparison between conventional diesel and Syntroleum fuel showed that average daily and, therefore, cumulative carbon dioxide production was much higher for Syntroleum than for diesel in fertilized soils under different temperature and contaminant levels (Figures 1, 2, 5, and 10). In the extended four–month experiment at 20°C, a 50% higher cumulative amount of CO_2 was produced for Syntroleum fuel as

compared with diesel, and at 6° C the CO_2 amount for Syntroleum was three times that of diesel (Figure 3). One of the main differences between diesel and Syntroleum fuel is the percent of aromatics in their chemical composition. As compared with diesel fuel, Syntroleum has minimal or no detectable aromatics, is consequently less toxic, and has fewer long-chain hydrocarbons (Bergin, 2006, Pers. Comm.). Aliphatic hydrocarbons, especially short-chain ones, degrade faster than aromatics (Wrenn and Venosa, 1996; Braddock et al., 2003), which is one of the main reasons for achieving higher microbial degradation with Syntroleum. The carbon shown as "other," which was not accounted for by these measurements, is a combination of carbon assimilated in biomass, carbon incompletely degraded, and carbon evaporated to the atmosphere. The hydrocarbon loss from soil can be as much as twice the value calculated from the CO_2 data which does not account for volatilization, long-term sorption, and incorporation into new biomass (Van De Steene and Verplancke, 2007). The recovered DRO in the soil by the gas chromatography may have been underestimated because the first few minutes of the spectrum were discarded due to interference with the solvent used. According to an Energy Laboratories, Inc. report (2005) on conventional diesel, the light carbon compounds, which are more volatile, are between 8% and 10.9% of total hydrocarbons.

2.4.2 Effect of temperature on respiration rates

Temperature plays a significant role in bioremediation of hydrocarbon-contaminated soils. The respiration rates and O_2 consumption of cold adapted microorganisms increase with rising temperature (Atlas and Bartha, 1998; Walworth et al., 2001; Ferguson et al.,

2003b). For microbial cultures in an arctic and subarctic environment, a temperature range of 1°C to 25°C can be assumed normal based on general summer air temperatures of Interior-Alaska. This part of the study investigated the respiration rates at two constant temperatures: 6°C and 20°C in fertilized soil. Figure 2 depicts the daily CO_2 respiration data, that is, the amount produced each day (rather than the total CO_2 generated) over a 17-week period. After approximately 85 days, the amount of CO_2 produced was low and continuously declining; therefore, the later data is not included in the figure. At a temperature of 20°C, microbial activities were highest only a few days into the experiment. At 6°C it took an average of three weeks for the microbes to adapt to the colder environment and peak in respiration rates. However, the highest daily CO_2 production was much lower compared with that at high temperature. On the other hand it was a much longer time before the respiration rates started to decline at the lower temperature (Figure 2). Figure 3 shows the average distribution of carbon present in the soil after incubation. In general, 40–60% of the added hydrocarbon was recovered by the two methods (CO_2 and GC/MS) after 17 weeks. Syntroleum mineralization was 2.5 times greater at the higher temperature, compared with the lower temperature, and diesel mineralization was almost 5 times greater at the higher temperature (Figure 3). During the first four weeks, a significantly higher Syntroleum degradation rate was observed at the higher temperature. It is generally accepted that the reaction rate doubles for each 10° C increase in temperature (Clark, 1996). This statement is not applicable, however, for hydrocarbon removal rate constants. Walworth et al. (2001) found that diesel biodegradation rates increased 4.8 times when the temperature increased from 1°C to

11°C and 1.8 times when the temperature increased from 11°C to 21°C in subarctic soils. Figure 4a shows the natural logarithm of the remaining carbon concentration (initial amount minus CO_2 produced) versus time. For a first-order reaction, the data should fall on a straight line whose slope corresponds to the rate constant as apparent from equation (2). In the case of Syntroleum the overall biodegradation rate constant was approximately 1.4 times higher (from $0.0045d^{-1}$ to $0.0066\ d^{-1}$) when the temperature increased by 14°C (Figure 4a). However the rates that were used in this calculation represent the overall rates for the 17–week period, which decreases significantly when the substrate becomes limiting. For the lower temperature a good r^2 value of 0.98 was achieved, indicating that the first-order model fit well. For the higher temperature, the data did not fall on a straight line, and the r^2 value was only 0.88. Figure 4b shows the cumulative CO_2 production along with model predictions. The actual experimental data clearly show different phases, indicating that it would be more appropriate to consider different rates in different growth phases due to changing microbial activity (Figure 4b). When rate constants were separately determined for different growth phases, the differences between the two temperatures became more apparent with much higher rates at higher temperature during the exponential growth phase ($0.0064\ d^{-1}$ for 6° C versus $0.0191\ d^{-1}$ for 20°C for Syntroleum). In addition to the experimental data, Figure 4 shows model predictions for the remaining concentration over time, based on three growth phases. At 6°C, the model has an excellent fit and is virtually indistinguishable from the data when three phases with different k values are assumed, and the fit is still good if only one overall constant is used. At 20°C, where the CO_2 production fluctuates more strongly, a

good fit is obtained with the three-phase model; a poor fit occurs when only one overall constant is used. In duplicated experiments at 6°C and 20°C, the average rate constants of the duplicates varied usually by about 10% (or less than 0.0005 d^{-1}) from each other. If rate constants vary by more than this amount, as it was the case for the different temperatures (Table 1), the difference between the k values was therefore considered significant.

2.4.3 Effect of nutrient levels on degradation

The soil nutrient concentration is another major factor that influences the degradation process. Two sets of experiments were conducted with nutrient contents of 300 mg N/kg or 0 mg N/kg respectively. Analysis of the original soil nutrient concentration showed some detectable but very minimal quantities of nitrogen, ammonium, and phosphorous in the soil. It was concluded, therefore, that the soil was nutrient deficient based on extractable nitrogen values. Too low or too high nitrogen in the soil can inhibit degradation processes (Ferguson et al., 2003a; Walworth et al., 2003). In this study, 300 mg N/kg were used, because this amount had been determined to be the optimal nutrition addition by an earlier study conducted by Schiewer and Niemeyer (2006). After nine weeks of treatment, the fertilizer addition had increased the amount of CO_2 produced from Syntroleum at 20°C by a factor of 3, as compared with the soil that had low natural nutrient content (Figure 5) and by a factor of 8 for the same conditions and contaminant at 6° C (data not shown). To illustrate the effect of nutrient addition, Figures 5 and 6 show the general results at different times of the experiment. Averaging for the whole 17-

week study, the overall respiration rate constant without nutrient addition is approximately 36% of the respiration rate constant achieved with addition of fertilizer for Syntroleum, with k values of 0.0023 d^{-1} without nutrient addition and 0.0066 d^{-1} with nutrient-sufficient soil (Figure 7). However, if a shorter time period is taken into consideration, the difference increases significantly, as evident from Figure 5. Especially in the second phase, the rate constant with nutrient addition (k=0.0191 d^{-1}) is almost 8 times higher than without (k=0.0025 d^{-1}) (Table 1). Figures 5 and 7 show the model predictions considering three growth phases for the fertilized and unfertilized Syntroleum-contaminated sample. For the unfertilized sample, the model fit is excellent (again, the model is difficult to distinguish from the data); for the fertilized sample, a good fit is obtained. For diesel fuel, fertilizer addition also increased respiration; however, the difference between fertilized and unfertilized samples was not as pronounced as for Syntroleum. As shown in Figure 6, fertilizer increased cumulative CO_2 production by 119% for Syntroleum compared with 76% for diesel.

In general, predictions by the three-phase model were much better than those using overall rate constants. The last two columns of Table 1 show the root mean square errors (RMSE) between experimental data and model predictions. When one overall rate constant was used, the average RMSE value was 0.0328, which was almost four times as high as for the three-phase model, which had an average RMSE of 0.0086.

2.4.4 Effect of moisture contents on respiration rates

Different gravimetric moisture contents (2%, 4%, 8% and 12%) were applied to Syntroleum-contaminated sand, conducted in soil fertilized with 300 mg N/kg and incubated at 20°C (Figure 8). Due to the physical characteristics of this type of sand, more reliable data can be obtained in small-scale experiments than can be obtained with gravel, which is less homogenous (data not shown). Soil moisture distribution can have an important influence on degradation rates (Davis et al., 2003). To investigate this effect, additional water was applied to the soil surface while ensuring that no significant evaporation occurred in the setup. It has been noted in other studies that 10% water saturation (which would correspond to 2.5–3.2% gravimetric water content for the sand used here) might reduce microbial degradation to an insignificant level (Saadoun and Al-Ghzawi, 2005). Previous studies have also noted that and 20–80% of soil saturation (which would be 5–20% gravimetric water content for the sand used here) should be sufficient for achieving increased biodegradation (Bossert and Bartha, 1984). Ferguson et al. (2003a) investigated the effects of soil moisture differences on hydrocarbon mineralization rates by using 9.9% to 39.8% gravimetric moisture content, and concluded that the water content did not show a major impact on hydrocarbon removal. Similarly, in this experiment the chosen moisture contents in sand proved to be only a minor factor, as shown in Figure 8.

2.4.5 Effects of frequent mixing on biodegradation

Mass transfer limitations (for oxygen and nutrients) can have a negative effect on degradation rates. To investigate whether this was the case, several batch experiments were conducted where daily or bi-daily agitation was applied for samples with 4% and 8% initial gravimetric water content (Figure 9). Soil with relatively low water content should not limit oxygen diffusivity during the batch scale studies (Rayner et al., 2007). Although the initial CO_2 production with the soil agitation was higher than in the other experiments (data not shown), after a few weeks this trend became less significant. By the end of the experiment, similar results were obtained without intensive soil agitation (Figure 9), which may imply that the O_2 transfer into the soil for microbial activities was not a limiting factor in the preceding setups.

2.4.6 Effect of soil types and different contamination levels on degradation

The effects of the contamination levels and soil types were investigated for 12 weeks (Figures 10a and 10b). In general, the doubled contaminant level (2000 mg vs. 4000 mg fuel/kg soil) led to an approximate doubling in the cumulative mineralization of the hydrocarbons for both soil types (Figure 10). As shown in Figure 1 and Table 1, the rate constants were virtually identical for low- and high-contaminant dosages: $0.0041 d^{-1}$ vs. $0.0043\ d^{-1}$ for Diesel and $0.0066\ d^{-1}$ vs. $0.0064\ d^{-1}$ for Syntroleum. Similar study performed with different diesel contamination levels, the author found less difference in the cumulative respiration data for high and low contamination levels (Niemeyer, 2003); however there were several differences in the soil characteristics between the two studies.

During the investigated time period, the contaminated sand showed twice as high cumulative CO_2 production as that of the contaminated gravel. Gravel has little particle specific surface area, limiting its ability to retain contaminants. This is in contrast to the sand, where adsorption process slows the contaminant movement vertically, resulting in more uniform contaminant distribution within the soil. The larger surface area of sand also enhances the availability of the contaminant to microbial cultures and consequently results in a higher hydrocarbon removal rate than gravel (Figure 10). Additionally, the sand had a larger porosity, which may have led to better oxygen supply.

The comparison of the fuel types shows that the diesel-contaminated soil generated between 60% and 70% of total CO_2 production of the total Syntroleum–contaminated soil for both contaminant concentrations and soil types (Figure 10).

2.5 CONCLUSIONS

Synthetic diesel fuel showed significant to marginally higher degradation rates in all aspects of this study as compared with conventional diesel fuel. The highest degradation of the hydrocarbons was around 75% total mineralization of the contaminant during four months. The mass balance could not be 100% closed based on measurements of CO_2 production and DRO remaining in the soil. The remaining carbon attributed to volatilization, incomplete degradation and conversion into new biomass. The bioremediation process started much earlier at higher temperatures compared with lower

ones, though microbes adjusted to the lower temperature and degraded the hydrocarbons to a significant extent. With sufficient nutrient supply for the microorganisms in the soil, the degradation of the contaminant after 17 weeks was almost 3 times higher at 20°C and 8 times higher at 6°C for the Syntroleum compared with nutrient-deficient sands. The investigated moisture contents (2–12% by weight) and regular mixing proved not to be strong factors affecting respiration rates. Interestingly, doubling the contamination levels from 2000 mg/kg dry soil to 4000 mg/kg dry soil resulted in a doubled cumulative CO_2 production. Regarding the soil types, contaminated sand showed close to 100% higher cumulative CO_2 production than contaminated gravel under identical conditions. First-order degradation rate constants were determined either for the overall experiment or for the three distinct growth phases. A model using three different rate constants was able to accurately model the development of CO_2 production over time.

2.6 ACKNOWLEDGEMENTS

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FIGURES

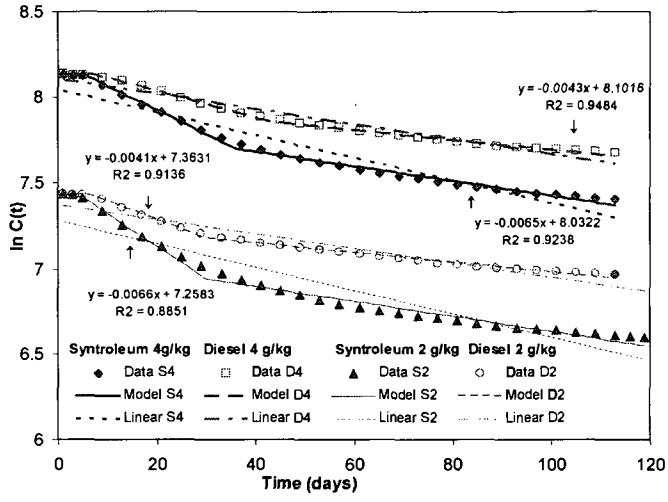


Figure 2.1 Linearized first-order plot for determination of the overall respiration rate constant using the integral method for Syntroleum and diesel fuel. Conditions: 20°C, 2000 mg/kg or 4000 mg/kg Syntroleum versus 2000 mg/kg or 4000 mg/kg diesel, 300 mg N/kg, sand.

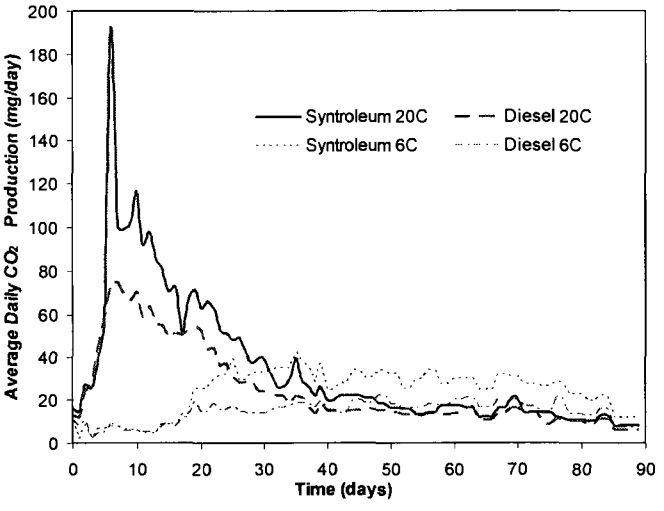


Figure 2.2 Syntroleum versus diesel daily respiration rate. Conditions: 20°C (20C) versus 6°C (6C), 2000 mg/kg of Syntroleum and diesel, 300 mg N/kg sand.

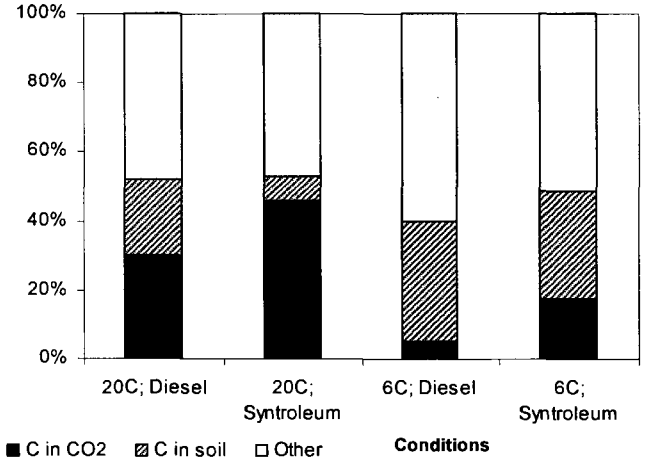
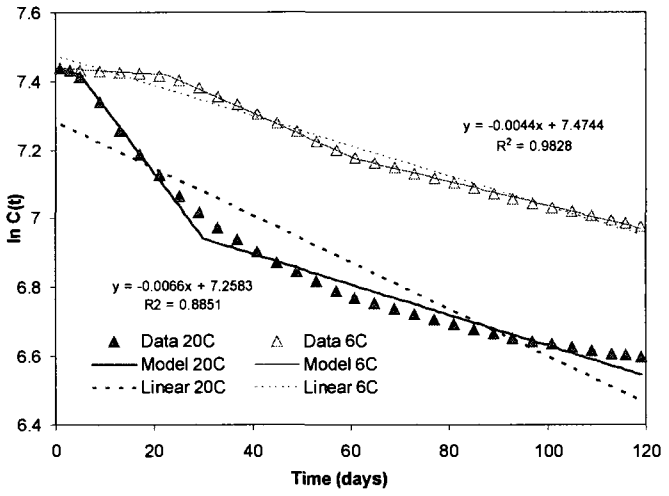


Figure 2.3 Percent carbon mass balance for different fuel types and temperatures. Conditions: 20°C (20C) versus 6°C (6C), 2000 mg/kg of Syntroleum and diesel, 300 mg N/kg sand.Experimental duration 17 weeks.

a)



b)

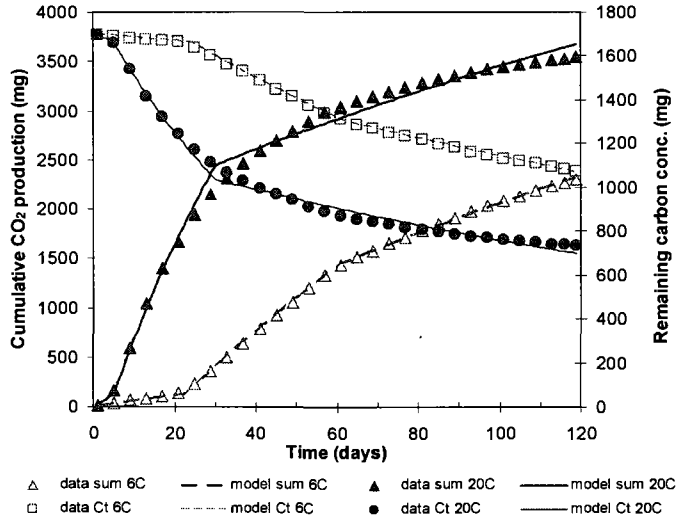


Figure 2.4 a) Linearized first-order plot for determination of the overall respiration rate constant using the integral method for Syntroleum at different temperatures; b) Cumulative CO_2 production (sum) and remaining carbon (Ct) for Syntroleum at different temperatures. Conditions: 20°C versus 6°C, 2000 mg/kg of Syntroleum, 300 mg N/kg, sand.

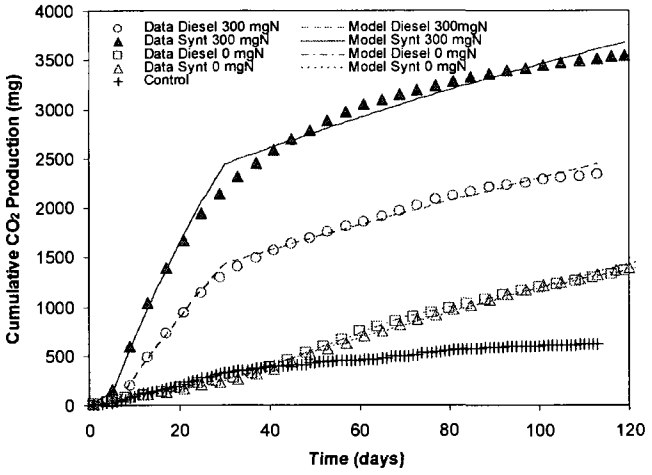


Figure 2.5 Cumulative CO_2 production at different nutrient levels. Conditions: 20°C, 2000 mg/kg Syntroleum and diesel, 300 mg N/kg versus 0 mg N/kg, sand. The control line represents the soil without contaminant and 0 mg N/kg.

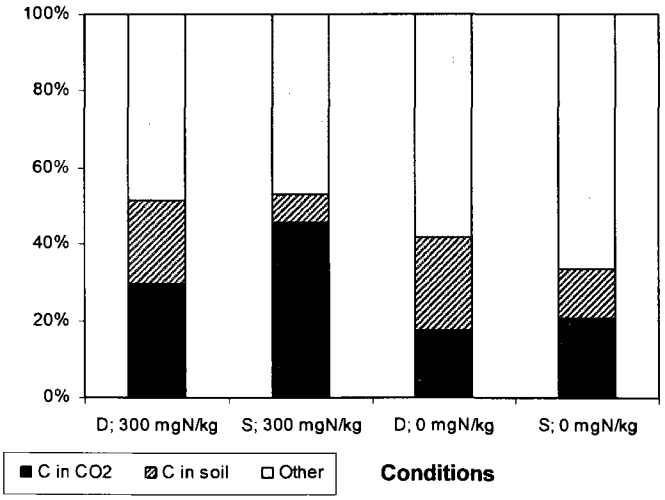


Figure 2.6 Carbon mass balance for different fuel types and nutrient levels. Conditions: 20°C, 2000 mg/kg of Syntroleum (S) or diesel (D), 300 mg N/kg versus no additional nitrogen, sand. Experimental duration 17 weeks.

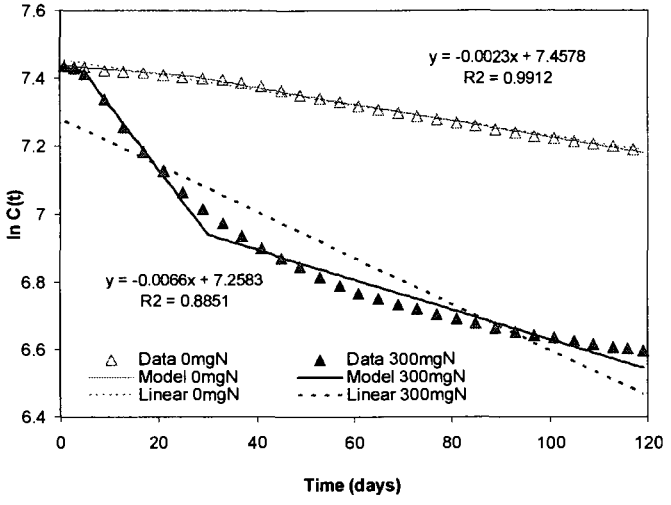


Figure 2.7 Linearized first-order plot for determination of the overall respiration rate constant using the integral method for Syntroleum at different nutrient concentrations. Conditions: 20°C, 2000 mg/kg of Syntroleum, 300 mg N/kg versus 0 mg N/kg, sand.

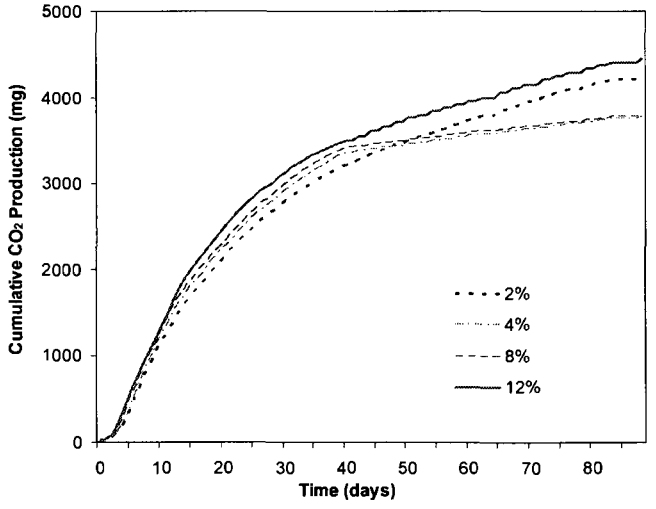


Figure 2.8 Cumulative CO_2 production for different soil moisture contents. Conditions: 20°C, 2000 mg/kg of Syntroleum, 300 mg N/kg, sand, 2, 4, 8 and 12% gravimetric water content.

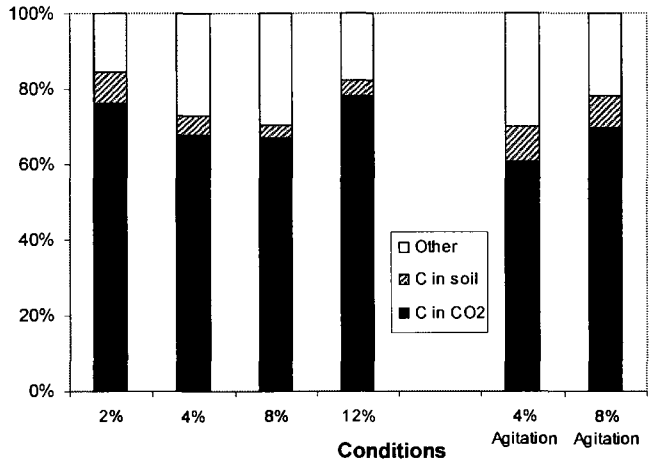
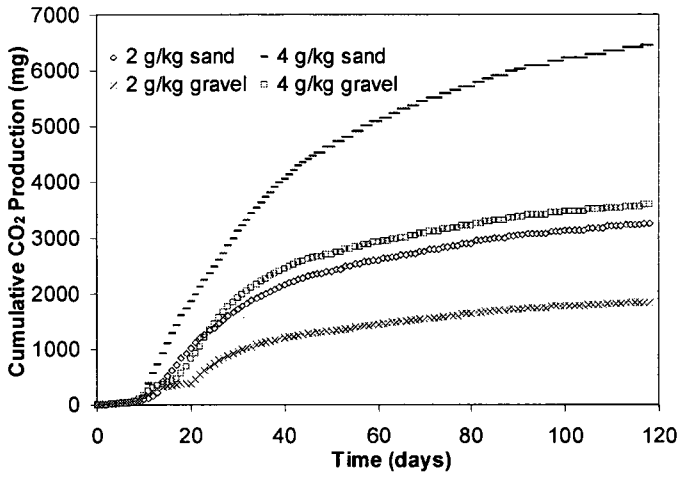


Figure 2.9 Carbon mass balance for different soil moisture contents with or without daily agitation. Conditions: 20°C, 2000 mg/kg of Syntroleum, 300 mg N/kg, sand, 2, 4, 8, and 12% gravimetric water content. Experiment duration 17 weeks.

a)



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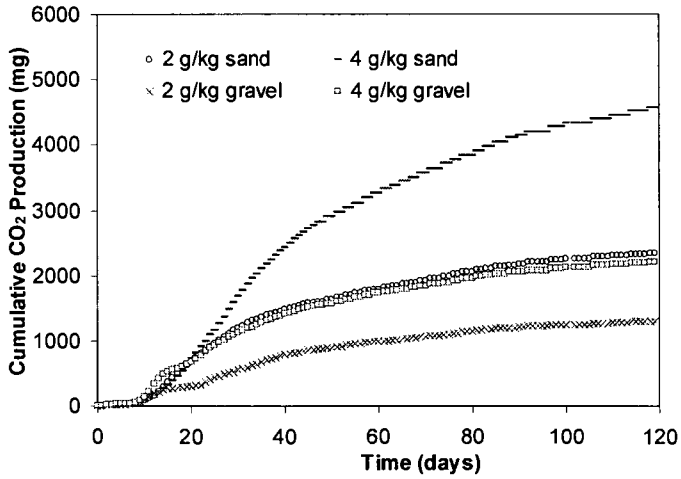
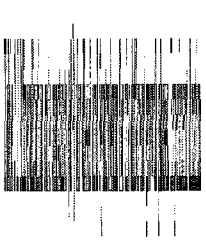
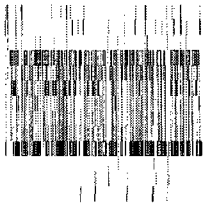


Figure 2.10 a) Cumulative CO_2 production for different contamination levels for sand; b) Cumulative CO_2 production for different contamination levels for gravel. Conditions: 20°C, 2000 mg/kg versus 4000 mg/kg of Syntroleum (S) or diesel (D), 300 mg N/kg.





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3 Investigation of microbial growth phases and inocula for degradation of diesel, Syntroleum, and fish biodiesel contaminated Alaskan sand[1]

3.1 ABSTRACT

Biodegradation of hydrocarbon fuels by indigenous microorganisms in the Arctic and subarctic environment is a relatively slow process. When a fuel spill occurs, the first few weeks, where the adaptation of the microbes to the new substrate takes place, are crucial in the remediation progress. The faster the microorganisms in the soil adapt to the changed environment, the more complete the degradation of the substance in a limited time frame. The main objective of this study was to investigate microbial adaptation times to different hydrocarbon fuels as substrates. Interior Alaskan, largely homogenous sand was contaminated with different fuel types; conventional diesel as a control substance, arctic grade Syntroleum, and two differently processed types of fish biodiesel. The study investigated the adaptation period (lag times) and different growth phases (exponential and stationary/death) of the microorganisms for the specific types of fuels under optimal conditions. The study also focused on different microbial culture types already adapted to various hydrocarbon sources. The respiration rate (CO_2 production)

[1]Horel, A. and S. Schiewer. 2009. Investigation of microbial growth phases and inocula for degradation of diesel, Syntroleum, and fish biodiesel contaminated Alaskan sand. Prepared for submission in Environmental Science and Technology.

was measured as an indicator of microbial activity and contaminant mineralization, complemented by analysis for hydrocarbons at the end of the experiment by using Gas Chromatography/Mass Spectrometry.

For determining the length of growth phases, the authors propose using nonlinear parameter optimization, which is less subjective and more widely applicable than other methods. The length of the microbial growth phases varied significantly for the different fuel types. The lag phase ranged between zero and 17 days for optimal conditions and over 40 days in unfertilized samples. Microbes grown in soil contaminated with Syntroleum and processed fish biodiesel fuels showed the shortest adaptation times and highest daily degradation rates in contrast to diesel and raw fish oil. Carbon dioxide production was up to three times higher for Syntroleum compared to conventional diesel fuel. Short adaptation times usually were followed by a short but extensive exponential growth phases, where the largest degradation rate constants were observed. Degradation during the first weeks was generally higher if an inoculum of microbes adapted to a specific fuel type was used; however all fuels were degraded regardless of the type of inoculum and the importance of the inoculum type diminished during later weeks. A simple first order model with different rate constants for lag phase, exponential phase and stationary/death phase was sufficient to describe the data very well; it was not necessary to consider Monod saturation kinetics or biomass growth in the modeling.

3.2 INTRODUCTION

In the Arctic and subarctic environment, biodegradation can be one of the most appropriate remediation processes. In the latest years alternative fuels are becoming more widely used with increasing demand for renewable energy sources. When fuel is spilled in rural areas, where excavation and shipping to specialized treatment facilities is cost prohibitive, natural biodegradation might be the best and cheapest removal option. The research studied the degradation of different hydrocarbon fuels by naturally occurring microorganisms in Interior Alaskan contaminated soils. Several laboratory tests have shown relatively high total hydrocarbon degradation during a short period of time such as 27% of diesel degradation after one day in an aquatic environment (Zhang, Peterson et al. 1998) and up to 80% during 38 days in the terrestrial subsurface (Margesin, Hammerle et al. 2007), while some field studies showed much less degradation over a longer time period, even after years (up to 68% during 447 days) (Margesin and Schinner 2001). Good correlations between laboratory and field studies have been reported in some cases; however, diesel contamination tends to persist in the environment more than in the laboratory (Zytner, Salb et al. 2001). When cultivated bacterial cultures from a laboratory environment are applied in Alaskan soil, slower degradation might be observed due to the microorganisms competing for the substrate with indigenous species (Leahy and Colwell 1990; Atlas and Bartha 1998). When bacterial culture is cultivated with microorganisms from Alaskan soil, the lab results can be more applicable to the field. Microbial degradation of hydrocarbons in laboratory studies in cold environments might be

underestimated compared with field studies as some research suggests (Hohener, Dakhel et al. 2006).

In this research, arctic grade Syntroleum was used, which was produced from natural gas and processed through the Fischer-Tropsch gas-to-liquid technique by the Syntroleum Corporation. A main advantage of the ultra-clean synthetic fuel (Syntroleum) compared with conventional diesel fuel is that it is less toxic due to its minimal content of aromatics, sulfur, and heavy metals (FTA 2007). While this type of fuel is currently fairly expensive to produce compared with conventional diesel fuel, in the nearest future it might become more competitive to diesel (Bergin, 2007, Pers. Comm.). Syntroleum can be used in its pure form or as a blend with conventional diesel fuel without any mechanical modification to an existing diesel engine (FTA 2007). Since Syntroleum use in cold climates is still in the pilot phase, its environmental effects and biodegradability are less known. However Syntroleum is expected to be more degradable compared with conventional diesel fuel due to its higher content of straight aliphatics (C_{8-28}), which are typically more easily degradable than branched or cyclic hydrocarbons occurring in conventional diesel fuel. Prior research of the present authors demonstrated faster degradation of Syntroleum compared with diesel fuel (Chapter 2).

Prior to 2004, Alaska generated 8 million gallons of fish oil per year as a byproduct of the Alaskan fish industry (Steigers, Seshadri et al. 2004), which increased up to 13 million gallons by 2007 (AEA 2007). Two types of fish biodiesel were investigated in this study.

One was raw fish oil, which came directly from an Alaskan based fish processor without any processing. The second type was processed fish biodiesel, where the raw fish oil was shipped to Hawaii for conversion to biodiesel, through the transesterification process by a commercial biodiesel plant and shipped back to Alaska for study of its cold weather performance (Schmid, 2008, Pers. Comm.,) (Witmer and Schmid 2008). The fish oil biodiesel samples were comprised of biodiesel that had been degraded by oxidation and oxidized biodiesel that had been rehabilitated. The rehabilitation of the biodiesel consisted of adding 400 ppm by volume of the antioxidant, ethoxyquin. After the addition of the antioxidant, the biodiesel was passed through a bleaching column of clay to remove fats and oxidized components (Schmid, 2009, Pers. Comm.). The purpose of the treatment was to enable good engine performance and also lessen the chance of engine failure. The proposed application of the fish biodiesel is for use as a heating fuel in rural Alaskan communities, mixed with high sulfur diesel fuel. Collection of the raw fish oil is being organized from different parts of Alaska, from where it could be transported in large qualities from 2009 onwards (AEA 2007). The effects of the fish oil and fish biodiesel on the environment (e.g. on soil permeability) and its fate needed to be investigated due to the unknown consequences in the extreme surroundings.

Previous exposure to hydrocarbons has an important effect on the microbial communities' ability to degrade the substrate in the environment at a higher rate, compared with no prior exposure (Leahy and Colwell 1990). Addition of natural microbial cultures from Alaskan soils contaminated with a specific fuel enhances

biodegradation effectiveness for that fuel type and significantly shortens the adaptation time to the substrate (lag phase). Previous research investigated the adaptation periods when a new substrate was introduced to an uncontaminated soil (Horel and Schiewer 2009c). In the present research cultivated microorganisms from already contaminated soils were introduced to a specific substrate. Part of this experiment's main purpose was to see if the lag phase can be eliminated or considerably shortened by indigenous microorganisms already adapted to the specific fuel type compared with earlier studies where the inocula came from the same fuel source. To better asses the biodegradation effectiveness and predict the time period required to achieve satisfactory contaminant removal, modeling of degradation rates is necessary. Modeling of biodegradation is an important part of any microbial remediation process. Rate constant determination was performed using an exponential decay expression based on first order kinetics, where the degradation rate is assumed to be proportional to the remaining substrate, i.e. fuel concentration. Cultivated microbial inocula were added to contaminated soils to investigate adaptation times for remediation purposes. Using naturally occurring microorganisms, microbial growth phases were investigated, and degradation rate constants were determined.

3.3 MATERIALS AND METHODS

3.3.1 Methods

The rate of hydrocarbon degradation was studied as a function of time while varying the fuel type, contaminant dosage (2000 versus 4000 mg/kg dry soil), temperature (6°C versus 20°C), nutrient dosage (0 mg N/kg versus 300 mg N/kg), and inoculum types. This work was based on small-scale experimental studies of biodegradation of four types of fuels (conventional diesel, Syntroleum, raw fish oil, and processed fish biodiesel).

For each experiment, 1 kg of soil (sand) was placed in an airtight 2.5 L container. Quantified amounts (2000 mg/kg dry soil) of the chosen contaminant, arctic-grade Syntroleum, raw fish oil, fish biodiesel, or conventional diesel (#2), were added to the surface of previously uncontaminated soil. Additionally, a small amount of soil containing allochthonous microorganisms from a prior contamination site was added. In the first set of experiments, this was contaminated soil from an Interior Alaskan automobile reclamation center. In later experiments, soil from previous experiments containing microbial cultures already adapted to different fuel hydrocarbons was added to provide an inoculum of microbes. Fertilizer of the type 20–20–20 ($N–P_2O_5–K_2O$, where the total nitrogen ingredients were: 20% ammoniacal, 30% nitrate and 50% urea nitrogen) was dissolved in water with a final concentration of 30 mg N/ml of solution, and 10 ml of this solution was sprinkled over the soil surface, achieving nutrient dosages of 300 mg N/kg dry soil. For control experiments with addition of 0 mg N/kg, 10 ml of water were added to attain the same water content of the soil without additional nutrients.

The soil was not mixed after or during these additions to better represent natural conditions after a spill on the soil surface. The samples were incubated either at a constant 6°C or 20°C, or a fluctuating temperature ranging between 6°C and 20°C. For most experiments, some water was added, maintaining a gravimetric moisture content fluctuating between 4 and 6%.

Data were collected over different time periods, ranging from 4 weeks to 17.5 weeks. Carbon dioxide is the main product generated when a substrate is completely mineralized in aerobic degradation processes. Therefore, the respiration rate, that is, the rate of CO_2 production, was measured as an indicator for microbial activity. Typically, the container was opened once daily for re-aeration and to measure the evolved CO_2, which had been captured in 20 ml of 1N NaOH solution (Page, Miller et al. 1982). During the daily analysis of the small-scale experiment, CO_2 measurements were taken by using a method and formula developed by Stotzky (Page, Miller et al. 1982). The carbonate formed was precipitated with a 0.3 N $BaCl_2$ solution.

To relate carbon dioxide production quantitatively to the degradation of hydrocarbons, the following stoichiometric equation for CO_2 production was used, assuming a C:H ratio of 1:2.12 for Syntroleum (Bergin 2004):

$$CH_{2.12} + 1.53\ O_2 \rightarrow CO_2 + 1.06\ H_2O. \qquad \text{eq. 3.1}$$

This stoichiometric equation applies only to mineralization of the fuel. The amount of CO_2 measured as a result of microbial consumption of hydrocarbons might be different

from the actual hydrocarbon decrease in the soil. Use of this equation to estimate the amount of fuel degraded based on the amount of CO_2 produced can lead to over- or underestimations. The amount of fuel degraded may be underestimated based on CO_2 measurements, since not all substrate is completely mineralized, some being incompletely degraded or used for the production of new biomass. On the other hand, the total fuel degraded may be overestimated if some CO_2 is produced from other carbon compounds originally present in the soil. That scenario is unlikely in the present study since soils with low organic content were used. The predominant importance of the added fuel compared to other possible substrates was confirmed by the use of controls without fuel addition. A baseline was developed based on uncontaminated soil samples for comparison with the calculated CO_2 production of the degradation experiments.

First-order degradation rates were determined by using the integral method based on the calculated amount of substrate still available according to respiration data,

$$\ln C_t = \ln C_0 - k\,t \qquad \text{eq. 3.2}$$

where C_t is the remaining contaminant concentration at any given time t (based on respiration data); C_0 is the initial contaminant concentration; and k is the rate constant. According to this equation, in a plot of $\ln C_t$ versus time, the data points should fall on a straight line with the slope $-k$ if first-order kinetics apply, that is, if the rate of degradation is proportional to the remaining amount of contaminant at any given time. The rate constant can be easily obtained from the slope of a regression line.

To estimate the number of total hydrocarbon degrading bacterial cells originally present in the soil, the most probable number technique was used. Triplicate 1 g samples of uncontaminated, diesel-contaminated, or Syntroleum-contaminated soil that were used as inocula were suspended in 10 ml 1% wt/vol sodium pyrophosphate ($Na_4P_2O_7$). To each well of 96-well microtiter plates, 180 µl of Bushnell-Haas broth growth medium, 20 µl soil suspension, and 5 µl number 2 diesel fuel (for diesel-contaminated and clean samples) or Syntroleum (for Syntroleum-contaminated and clean samples) were added as a pure carbon source. The total hydrocarbon-degrader MPN plates were incubated for 2 weeks at room temperature before being scored for positive growth by using INT dyes to detect presence of respirating bacteria (Haines, Wrenn et al. 1996; Wrenn and Venosa 1996). Actual values were determined by using a corrected MPN table (de Man 1983).

To evaluate the relationship between substrate use and CO_2 production, gas chromatography/mass spectrometry (GC/MS) analysis was performed (Agilent Technologies 6890N Network GC System coupled to a 5873 mass selective detector with column parameters: 30 m by 250 µm by 0.25 µm) and compared to respiration data. For this purpose, the diesel range organics (DRO) determination was conducted using a modified method that was developed based on the AK 102 (ADEC 2002) and EPA 8270 (semi volatile organics by GC/MS) methods (ADEC 2002). The GC/MS DRO method used a spitless injection with helium carrier gas (pressure: 6.895 kPa; flow: 0.5 ml/min; average velocity: 26 cm/sec). The oven temperature started from 40°C till 320° C for the duration of 52.33 runtime. Recovery data analysis was performed for determination of the

actual hydrocarbon recovery value by the gas chromatography method used. For this purpose, a known amount of fuel was added to the soil and extracted immediately. The quantity of the average highest recovered hydrocarbon amount for each fuel type, which varied between 74 and 100%, was taken as a reference value of 100% for the GC/MS data analysis instead of the theoretical value (calculated by using a generalized stoichiometric equation). This was done due to some interference with the solvent during the first few minutes of the GC/MS spectrum. The lower weight volatile compounds, in particular, are not reliably measured, since their peaks occur at a time when the solvent is still eluted.

3.3.2 Soil characteristics

The soil used in the experiments was not sterilized and thus contained some naturally occurring microbial cultures that survive extreme conditions (–50°C to 30°C). The purpose for using unsterilized soil was to further simulate natural degradation processes. General soil analysis was performed using the instrument DX 500 with PeakNet software for nitrite and nitrate analysis (high performance liquid chromatography) and the ASTM standard for sieving. Additional analysis for total nitrogen and total carbon concentration was done by using an elemental combustion system (Costech Instruments).

The pH of the soil was analyzed before the experiments, using a Mettler Toledo pH meter at 21.8°C. The soil pH value was between 7.07 and 7.25, which is optimal for hydrocarbon degradation (Saadoun and Al-Ghzawi 2005). The original water content

before the start of the experiments was negligible, less than 1%. The bulk density and porosity of the soil was determined by using the ASTM standard. The soil particles were ranging mainly between coarse to very fine sand with less than 0.3% of clayey material. The average bulk density was 1.4–1.6 g/cm^3 for sand. The average porosity of the sand was between 39.7 and 49.3% depended on the amount of clay present in the soil. The average percent of total available nitrogen in the soil was 0.0054% and the fraction of (total) carbon was 0.165%.

3.4 RESULTS

3.4.1 Microbial growth phases

During the course of the study, three major microbial growth phases were easily determinable based on the daily respiration data (Figures 1 and 2). Typically, those were the lag phase, the exponential growth and the stationary/death phase. Table 1 summarizes the average time for the main growth phases that were observed during the experiments and Table 2 lists average degradation rate constants for the phases determined by first order linear regression and also by nonlinear analysis. Each phase is explained below in detail. In hydrocarbon degradation experiments, the short–chained alkenes could be investigated separately because these compounds are highly toxic to many microorganisms; they can also evaporate fast into the atmosphere (Atlas and Bartha 1998; Horel and Schiewer 2009a) (Chapter 5); however in terrestrial spills the contaminant movement occurs vertically, which can prevent evaporative losses (Leahy and Colwell

1990). The intermediate chain length (C_{10}–C_{24}) can easily be used as a carbon source for the microorganisms and degrade the fastest (Atlas and Bartha 1998).

3.4.1.1 Lag phase

The lag phase describes the time period when the microorganisms are adapting to the new environment and the cell growth is very slow or minimal. During the start of each experiment, a small quantity of microbes was added. The initial long–term experiments included microbial colonies from a reclamation center, while in subsequent shorter experiments, cultures previously adapted to specific fuel hydrocarbons were introduced to the soil. This difference in the setups resulted in some differences in the determined lag phase periods (Table 1).

Sand particles of the soil used in this study are relatively large and have relatively few attached microbes compared with clay particles, which hold more moisture and organic matter (Lynch and Hobbie 1988). In soils with low organic matter, the approximate initial number of aerobic bacteria was determined as $0.28–16.73*10^6$/g (Yanagita 1990). The total hydrocarbon degraders in uncontaminated soil determined here by MPN were between $0.181–3.63*10^4$/g with an average value of $9.37*10^3$/g soil. With a low initial density of microorganisms in the soil, even with multiple cell divisions of the organisms, the substrate degradation is small and hard to detect (Alexander 1994).

Most published bioremediation studies (Huesemann, Hausmann et al. 2004; Borresen and Rike 2007; Horel and Schiewer 2009c) use an intuitive approach for evaluating the length of the lag phase or other phases without quantitative justification. The present authors consider this aspect important enough to warrant further consideration. There are several possible criteria for defining the end of the lag phase:

1) When daily respiration exceeds a defined threshold value
2) When daily respiration increases extensively above previous days
3) When 1st order rate constant increases as indicated by increasing slope in plot of ln C versus t
4) Such that a chosen mathematical model matches the data best

While the first criterion is simple to apply (e.g. using a threshold value for the daily CO_2 production of 12 mg/d for data for 20°C in Figures 2a–c), it may not be feasible to do that if daily respiration rates vary strongly for different experimental conditions (e.g. fuel types, nutrient level, temperature) so that it is not possible to define one such threshold. For example, as Figure 1e shows, data at 6°C never reach 12 mg CO_2/d. Furthermore, there may already be an exponential increase even at low absolute values.

Mohn and Stewart defined the lag phase as the time till 1% of the total hydrocarbon available is mineralized (Mohn and Stewart 2000); however when the environmental conditions vary greatly among experimental setups, similar difficulties could be experienced as earlier mentioned with fixed daily values.

The 2nd criterion is harder to define; it is to some extent arbitrary how a "considerable increase" is defined. However, the approach avoids the problem of having to define one common threshold value for all conditions as in the first criterion. For example, the first day of the exponential growth phase could be defined as the day when daily respiration starts to increase steadily, such that for at least three days, the daily respiration at any of these days is higher than for any of the preceding days. This approach can however not be applied if there are fluctuations (ups and downs) in the daily respiration data (such as shown in Figure 2c for the fish oil inoculum), or if the increase of respiration is limited to a very brief time period.

If growth phases are identified based on daily respiration data (according to the 1st or 2nd criterion), the impression is created that the death phase occurs immediately after daily respiration peaks, e.g. for Syntroleum after two weeks of experimental duration (Figure 2a). This decline, however, may not be caused by decreasing microbial activity but by decreasing substrate concentration and may lead to erroneous identification of the start of the death phase.

The third criterion avoids this problem by focusing on changes in the specific rate per remaining substrate, as indicated by changes of slope in a linearized plot e.g. for first order kinetics. For example, in Figure 1c, an increase of the slope can be observed around day 17 for 20°C. The length of the lag phase would therefore be 17 days. According to

this method, the exponential and stationary phase extends to the end of the 28 day experiment (Figure 3), even though daily rates decline after reaching a maximum as shown in Figure 2. This approach could similarly be applied for other kinetics (e.g. 2^{nd} order) as long as a linearized plot is available. This approach has the disadvantage that a kinetic model already has to be chosen before the lengths of the phases can be determined. Another disadvantage is that the identification of the time at which a change in slope occurs is still subjective.

The fourth criterion is similar to the third one, with the exception that instead of intuitively choosing a time at which the slope changes, a mathematical criterion is defined: for a pre-determined number of phases, the model parameters (rate constant and length of each phase) can be optimized all at once by minimizing the root mean square error (RMSE) between model and experimental data. This approach is the most general and does not rely on any subjective choice except for the type of kinetic model.

Table 1 lists the lag phase durations determined for different experimental conditions according different methods. The obtained results differed widely. At optimal conditions, Method 1 led to very short lag phases, especially when compared to method 3. At low temperature or in unfertilized samples, the results for different methods were somewhat more similar. In this study, for the purpose of modeling and determination of rate constants, the lengths of all phases were determined according to the 3^{rd} and 4^{th} method. That means the lag phase was assumed to extend from the first day of the experiment till

an increase of slope was observed in a plot of the logarithm of the remaining substrate concentration versus time (Figures 1c and 1d). This method resulted in a longer lag and exponential phase compared with methods 1 or 2 (which were based on daily respiration rates, Table 1), and the determination of different growth phases was more reliable and a better model fit was obtained with under all environmental conditions investigated in this study.

The duration of the lag phase as determined here also includes the time when the microbial activities are measurable but fairly small (Table 2). By comparing 58 experimental setups under favorable conditions (not all of which are discussed in the present study), 67% of the setups the lag phases were less than 7 days according to method 3, where the average lag phase rate constants for the three main fuel types were $k_S = 0.00405\ d^{-1}$, $k_D = 0.00185\ d^{-1}$, and $k_F = 0.0016\ d^{-1}$ at 20° C with 300 mg N/kg soil. The longest lag phase under optimal conditions (24 days) was determined for fish oil ($k_F = 0.0010\ d^{-1}$) (Table 3), conversely in the case of diesel and Syntroleum it was no longer than 12 days.

When conditions, such as temperature and nutrient dosage, were suboptimal, the adaptation period became much longer. At low temperature the lag phases (22 or 25 days, respectively, for Syntroleum and diesel) were about 4 times as long as for 20°C. Similar results were obtained by a study conducted by Borresen and Rike (2003), where a 20 day lag phase period was observed for a diesel contaminated arctic site at low temperature.

However, several setups, e.g. fish oil with nutrient deficient soil at 20°C or 6°C with nutrient sufficient soil, showed no lag phase and the measured respiration was high from the first day of the experiment compared with later days. Other hydrocarbon degradation studies noted missing lag phase at low temperature (Eriksson, Ka et al. 2001), though in the present study low temperature corresponded with longer lag times in most cases. However, in the case of raw fish oil at 6°C, degradation rates during the first week were higher than in weeks 2–4 (Figure 1d). One interpretation of the behavior is the occurrence of an exponential phase starting without any prior lag phase, followed by a lag phase and a second exponential phase. An alternative explanation would be a residual higher activity initially since the soil was at a higher temperature during the setup of the experiment. This second interpretation is in accordance with observations of Horel and Schiewer where relatively high activity remained present for several days after the temperature was changed to a lower value (Horel and Schiewer 2009b)(Chapter 4). This would mean that the initially high respiration is an artifact and the lag phase extended from day 1 to day 25 for fish oil at 6°C (Figure 1d). Low temperature rate constants during the lag phase were lower for raw fish oil than for Syntroleum or diesel ($k_S = 0.0011\ d^{-1}$, $k_D = 0.0010\ d^{-1}$, and $k_F = 0.0005\ d^{-1}$; Table 2).

The lag phase rate constants were small in nutrient deficient soil ($k_S = 0.0014\ d^{-1}$ and $k_D = 0.0014\ d^{-1}$), and the adaptation period of 5 weeks was even longer than at low temperatures (3–4 weeks). When no nutrients were added, the lag phases for Syntroleum and diesel were 7 times longer than for fertilized samples at 20°C (Table 1). For raw fish

oil and fish biodiesel, the data did not follow the typical scheme of lag, exponential and stationary/death phase but the lag phase was missing with $k_F = 0.0009\ d^{-1}$ (Table 2); however the processed fish biodiesel behaved similarly to diesel and Syntroleum, with a lag phase duration of only several days (Table 1) with much higher rate constant $k_F = 0.0030\ d^{-1}$ (Table 2). This phenomenon can be explained by the different chemical composition of fish oil and fish biodiesel before and after the transesterification process respectively. Raw fish oil oxidizes rapidly when contacted with air which changes the viscosity and density of the fuel and limits the contaminant movement and thereby substrate availability for the microorganisms deeper in the soil.

3.4.1.2 Exponential phase

In a typical batch bacterial growth curve, the lag phase is followed by exponential growth and later a declining rate of growth when substrate availability is reduced; however the cell density is still somewhat increasing. These growth phases are ultimately followed by a stationary and death phase where microbial numbers decline (Vaccari, Strom et al. 2006). Since no direct data for biomass growth were available, in this study it was assumed that the second phase (exponential and declining rate of growth) lasts until the slope in the first order kinetics plot (Figures 1 c and d) decreases (applying criteria 3 and 4 for growth phase determination). In most cases, the exponential phase had a similar or longer length as the lag phase. The length of the exponential phase with rapid substrate utilization varied significantly between different setups.

The shortest periods of exponential growth were observed when the environmental conditions were favorable (20°C, 300 mg N/kg soil) in which case the degradation rate constants ($k \approx 0.002 - 0.030\ d^{-1}$) and the daily respiration were the highest (Table 2). The exponential phase transitioned to the stationary/death phase after 2.5 weeks for diesel fuel or 3.5–5 weeks for Syntroleum from the start of the exponential phase when conditions were favorable (Table 1), compared to much longer time periods for less favorable conditions. Due to high microbial activity under favorable conditions, the daily respiration peaked earlier with a higher average value (over 180 mg CO_2/day) and the available substrate was quickly reduced, resulting in a decreasing daily respiration after only a short time. In these experiments the exponential growth phase was between 11 and 36 days (Syntroleum, diesel, and processed fish biodiesel). However, when trying to determine growth phases from the peak in daily respiration data without considering depletion of available substrate concentration (an approach that the current authors consider erroneous), this phase appears to last only 4 – 5 days until a peak in respiration is reached (Figures 2 a, b). This discrepancy demonstrates the importance of using proper methods for determining the lengths of growth phases.

At 6°C, the exponential phase is 3–5 weeks longer (for Syntroleum and diesel respectively) than at 20°C (Table 1), with a much lower rate constant ($k = 0.0031–0.006\ d^{-1}$) than at 20°C (Table 2). However, the smaller degradation rate constants for a longer exponential growth period can ultimately result in a significant contaminant decrease in the soil by microbial activities (Horel and Schiewer 2007). As discussed

above, for fish oil, classical growth phases were not observed but an initial fast phase was followed by a slow phase and a second fast phase. The second exponential phase could have been due to microbes starting to use a different substrate and/or due to a change in the microbial community. Fungi started visually colonizing on the samples after 10 days. However, it is plausible that the true exponential phase only started on day 25 and the initially high respiration was a carry-over from high activity at high temperatures during experimental setup (Chapter 4). Initially higher respiration was also noted for diesel and Syntroleum contaminated samples in the present study.

When no nutrients were added, the exponential growth phase was shorter than at low temperature but longer than for the fertilized samples, lasting 3.5 or 6 weeks respectively for diesel and Syntroleum (Table 1). Without fertilizer, degradation rate constants were only about ¼ of those observed with fertilizer (Table 2). Low availability of nutrients for the microorganisms in the soil inhibits growth and the degradation rate constants are small (Ferguson, Franzmann et al. 2003; Walworth, Woolard et al. 2003). As mentioned above, for raw fish oil in nutrient deficient soil the lag phase was missing (Figure 1). The peak of respiration was not reached during the 28 day experiment; however a decrease of slope was noted in the 1st order plot on day 17, so the duration of the exponential phase, without prior lag phase, was considered to be 17 days (Figure 1).

The exponential phase is typically characterized by an exponential growth of microbes and a concurrent exponential decline in substrate concentration. Therefore, a first order

model (equation 2), featuring an exponential decrease of the substrate concentration over time, was used. Figures 1c and 1d show determination of the 1st order degradation rate constants for the raw fish oil in a logarithmic plot. It can be observed that the data not follow one overall regression line very well; several distinct phases can be observed. Therefore, separate rate constants were determined for each phase as suggested by Horel and Schiewer (2009c), which greatly improved the model fit, especially when nonlinear parameter optimization using the Excel solver function was applied rather than a linear regression. Using the rate constants obtained by nonlinear optimization (Table 2), the remaining amount of hydrocarbons and the cumulative CO_2 production were modeled very well as shown in Figures 1a and b. Modeling based on Monod kinetics is discussed below (3.4.4).

3.4.1.3 Stationary and death phase

During the stationary phase, microbial respiration is still significant and microbial numbers are high; however the microbial population is steady or declining and substrate utilization rates decrease significantly. In this paper the stationary and death phase were combined since substrate utilization rates are low in either case and actual microbial numbers were not available. To study and understand the parameters of this phase is important for bioremediation purposes. Changes in environmental conditions, such as temperature increase or additional nutrients, may again accelerate the biodegradation process, resulting faster and more complete degradation. Under favorable conditions, where the rate constants are high within the first week of the experiment ($k > 0.01$ d^{-1}),

the substrate supply becomes limited during a short period of time, and cannot support the exponential biomass increase and the microbial growth starts to decline. This is the time when the shorter–chained hydrocarbons and other easily degradable compounds are already used up by the microorganisms and the longer–chained compounds and carbon from dead microbial cells become the main carbon source. Very long-chain alkenes from petroleum hydrocarbons are very resistant for biodegradation (Atlas and Bartha, 1998); however in the current study neither of the contaminant types has more than 0.1% of C_{24} or higher carbon chain (Alaska Energy Laboratories, 2005). However, it was noted in the experiments that during this growth phase the substrate use can be significant on a long-term (e.g. at low temperature) when the rate of respiration does not decline as fast as in the previously mentioned higher temperature examples.

3.4.2 Effect of previously adapted microbial cultures on fuel degradation

In contrast to experiments where all samples were initially inoculated with a small volume of soil contaminated with conventional diesel fuel to introduce a microbial population (Figure 1), an experiment was conducted where each sample was inoculated with a population that was already adapted to a specific fuel type. This was done by adding a small volume of soil with prior contamination of the respective fuel type. Thus a fair comparison of different growth phases under favorable conditions of 20°C and 300 mg N/kg sand was possible, where each sample had already an adapted microbial population present.

The measurable degradation for the Syntroleum contaminated samples started within less than a day for each inoculum type based on respiration data. The lag phase (where the degradation by the microbial activity is slow, usually less than 12 mg CO_2/day) for Syntroleum or diesel substrates lasted 1–2 weeks (Table 3). The four weeks experiment covering different microbial growth phases showed that for the first two weeks, Diesel fuel and Syntroleum had similar biodegradation trends, as shown in Figure 2; however with higher respiration for Syntroleum. After three weeks, Syntroleum had 2.3 times higher cumulative CO_2 production than the conventional diesel fuel (Figure 2d) and 4.7 times higher than raw fish oil, when the fuel specific inocula were used. The large difference in the degradation of Syntroleum and diesel can be explained as by Horel and Schiewer (2009c), where Syntroleum proved to be more easily degradable and consequently showed a larger cumulative CO_2 production.

Syntroleum and diesel were both degraded at a higher average rate than raw fish oil (Figure 2, Table 3). During the first two weeks raw fish oil showed little microbial activity (Figure 2) but by the end of week three the CO_2 production becomes very significant, which was appeared linked to fungal growth (data not shown). The roles of fungal growth in hydrocarbon metabolization are the subject of a separate investigation by the present authors (Chapter 5).

In most cases, cumulative CO_2 production and the daily respiration peak were highest and earliest when inocula of microbes that were already adapted to the respective contaminant

were added (Syntroleum degradation with Syntroleum-based inoculum, fish oil degradation with fish oil inoculum). This conforms to the hypothesis that previous exposure to a given fuel type leads to an optimally adapted microbial consortium and therefore to higher respiration.

The only exception was observed for diesel as a substrate, where respiration was higher using a Syntroleum- than a diesel-based inoculum. The daily respiration peak occurred earliest and was highest for the Syntroleum inoculum (Figure 2a). This can be explained by the difference in microbial densities in the inocula where Syntroleum-based inocula had initially a higher microbial activity than diesel-based inocula. When microbial numbers were determined by using the MPN technique, inocula from previously diesel-contaminated soil had 7 times lower average microbial numbers compared to Syntroleum contaminated sand ($1.57*10^4$ versus $1.05*10^5$ g^{-1} soil respectively). The lower microbial count in diesel-inocula also explains that degradation of Syntroleum and fish oil was lower for addition of microbes adapted to diesel than for other inoculum types (Figure 2d). After daily respiration for Syntroleum adapted inocula in the diesel contaminated soil reaches its peak, it decreases rapidly while the diesel-adapted microbial cultures steadily degrade the diesel fuel for a longer time period (Figure 2b) resulting in a similar cumulative CO_2 production by the end of the experiment (Figure 2d). By extrapolating the respiration trends, it is reasonable to expect that for a longer experimental duration the cumulative CO_2 production would be somewhat higher for the diesel adapted inocula compared with the Syntroleum adapted microbes (Figure 2d).

When raw fish oil was investigated, the microbial respiration was augmented by additional mineralization associated with fungal growth, where the visual colonization started 9 days after starting the experiment. This trend was continuously observable for all fish oil and fish biodiesel contaminated soils both at higher and lower temperatures (data not shown), and is further discussed in Chapter 5 (Horel and Schiewer 2009a). Additional respiration by fungal colonies, which were observed after day 10, may be the reason for the unusual acceleration of CO_2 production after day 26 (Figure 1b). An alternative explanation is that this was really the only exponential phase and initially high respiration rates occurred because of residual high activity from setting up the experiments at room temperature.

3.4.3 Fraction of hydrocarbons mineralized

The hydrocarbon peaks of GC/MS spectra were investigated to evaluate the disappearance of the different carbon chains from the soil. In general, for diesel fuel, the diesel range organics (DRO) chain length of C_{12}–C_{24} are in the range of interest. In this paper only this range was studied. Figure 4 shows the carbon mass balance at the end of the inocula study, assuming that all measured carbon dioxide was derived from mineralization of the fuel. Figure 1a shows that mineralization is very low when no fuel was added, with only 158 mg CO_2 produced in 40 days. The amount mineralized is highest for Syntroleum as the main substrate and decrease in the order Syntroleum > diesel > fish oil, regardless the type of inoculum. The amount of hydrocarbons recovered

in the soil on the other hand is generally highest for fish oil, and decreases in the opposite order, except for fish oil as main fuel versus diesel both using fish oil based inoculum. The total amount of carbon recovered ranged between 50 and 95%. This means some carbon is not directly accounted. It is only to be expected that fractions of hydrocarbons volatilize as described by Horel and Schiewer (2009a; Chapter 5) or are converted to biomass.

As Figures 1, 4, and 5 show, the first month has major importance in the microbial respiration process as the carbon source is being mineralized, forming carbon dioxide. The data in Figure 4 were obtained by relating the carbon present in the initially added fuel to the carbon present in the evolved CO_2. The percentage of hydrocarbons mineralized, based on the measured respiration, within a 6 week period was highest for Syntroleum with 32%, followed by fish oil with 24% and diesel with 22%.

As shown in Figure 5b, the highest degradation for Diesel and Syntroleum occurred during week 2, whereas for fish oil, degradation was maximal during week 3 and 4. Syntroleum had the highest hydrocarbon removal of 10.33% during the second week of degradation, followed by the second highest weekly degradation with 8.05% in week three. Compared to diesel, which achieved a maximum weekly degradation of 6.74% during week 2, fish oil showed a higher cumulative weekly hydrocarbon degradation of 7.40% during week 4 and 7.28% on week 3, respectively.

3.4.4 Choice of model

As shown in Figure 1 and according to RMSE in Tables 2 and 3, a simple first–order model fit the data surprisingly well when 2–3 phases were considered.

Based on typical microbial growth kinetics according to the Monod model; however a different behavior would have been expected, where rates are not proportional to the substrate concentration and where the biomass concentration also plays a role. The Monod kinetics, which describe the impact of the substrate concentration on the rates can be written as

$$\mu = \frac{\mu_{max}\ S}{K_S + S}\ , \qquad \text{eq. 3.3}$$

with μ the specific growth rate, μ_{max} the maximum specific growth rate, S the substrate concentration, and K_S the half saturation constant, i.e. the substrate concentration where half of the maximum rate is achieved. According to this model, the rate increases linearly with the substrate concentration for low substrate concentrations and levels off at a plateau value of μ_{max} for high substrate concentrations where $S >> K_S$.

It is commonly assumed that the microbial growth rate r_g is proportional to the substrate degradation rate r_S and to the rate of product formation r_p,

$$r_g = \mu X = - Y_{X/S}\ r_S = r_p / Y_{P/X} \qquad \text{eq. 3.4}$$

with X the biomass concentration, $Y_{X/S}$ the yield factor of microbes produced per substrate consumed, P the product (in this case CO_2), and the product yield factor $Y_{P/X}$.

Since the sum of biomass production $r_g = Y_{X/S}\, r_S$ and product generation $r_P = Y_{P/X}\, Y_{X/S}\, r_S$ cannot exceed the rate of substrate utilization r_S (all on a basis of mass of carbon), the term $Y_{X/S}\,(1+Y_{P/X})$ shall not exceed 1.

Model calculations were run with the aim of optimizing the parameters μ_{max} and K_S by minimizing the RMSE between model and data. However, no stable optimum was obtained. Closer inspection revealed that these parameters could not be optimized independently because the resulting fit only depended on the term μ_{max}/K_S, not on each of these parameters by themselves. This is consistent with low concentrations in the linear range of the Monod equation, where the rate depends linearly on the substrate concentration.

To determine whether that was indeed the case, experiments with initial substrate concentrations varying from 500 – 20,000 mg/kg were conducted. The results are shown in Figure 6. Since substrate concentrations varied not only for different initial concentrations but were also reduced over time, several data points are depicted for each initial concentration, representing the concentrations obtained by the end of each week. The data show that except for the first week, where rates, esp. at high concentrations, were lower due to lower lag phase rate constants, the data followed one common trend. All data for weeks 2–4 could be described with the Monod equation using K_S = 1728 mg C/kg dry soil and a maximum substrate degradation rate $\mu_{max}\, X/Y_{X/S}$ = 350 mg C/week shown as the solid line in Figure 6. Coincidentally, K_S corresponded very closely to the

initial substrate concentration of 1714 mg C/kg dry soil. As shown in Figure 6, for low substrate concentrations of approximately 1100–1700 mg C/kg (i.e. for most data of the present study) the relationship between substrate concentration and rate is approximately linear. This is supported by the finding that even for an initial concentration of 4000 mg fuel/kg, the rate constants were similar to those for 2000 mg/kg.

Attempts to consider increases in biomass concentration by simultaneous numerical integration of rate equations for substrate use and biomass growth, fitting μ_{max}, $Y_{X/S}$ and $Y_{P/S}$ while using the K_S value determined from Figure 6, were not successful and lead to too low maxima and too high rates during later weeks. If death of the biomass was also included in the model with the death rate constant k_d, to decrease rates in week 3 and 4, according to the equation $r_X = r_S\, Y_{X/S} - k_d\, X$ no good fit was obtained either.

Based on the above findings, it is reasonable to assume a constant biomass concentration and a linear relationship between substrate concentration and rate, as for the 1st order model, such that the above equations can be simplified as

$$r_S = -\frac{\mu X}{Y_{X/S}} = -\frac{\mu_{max}}{K_S + S}\frac{S}{Y_{X/S}}\frac{X}{} = -\frac{\mu_{max}\, X\, S}{K_S Y_{X/S}} = -kS. \qquad \text{eq. 3.5}$$

Since the first–order model is simpler, has a lower number of fitted model parameters, and provides a better fit than other model alternatives, it was concluded that first–order kinetics are the best model for representing the present data.

3.5 CONCLUSIONS

Biodegradation of hydrocarbons was fastest during the first few weeks of the remediation process. After 6 weeks, 31.74% of the Syntroleum fuel was already microbially mineralized under optimal conditions, which was 1.45 times higher than for conventional diesel (21.90%).

The lengths of growth phases should not be determined from daily respiration data but based on the fit of a suitable kinetic model. In this study, first order kinetics fit the data very well when separate rate constants were determined for 2–3 phases. The transition from one phase to the next was indicated by changes in the slope in a 1^{st} order plot of the logarithm of the remaining substrate concentration versus time.

Under optimal conditions, the lag phase was short; lasting only about a week or less for Syntroleum and diesel, and a pronounced peak of daily CO_2 production was observed between day 6 and day 15. For fish oil, each phase was longer. At suboptimal conditions all phases were extended and slow, steady metabolization continued over a longer time. This can be explained by the fact that under optimal conditions rapid cell growth and substrate mineralization occurs, until after only a short time easily degradable substrate is no longer readily available, thus limiting further degradation.

For fish biodiesel, the normal pattern of lag phase, exponential phase and stationary phase could not be observed when conditions were unfavorable. When no nutrients were

added, no lag phase could be discerned. At low temperatures, the daily respiration rate was initially fast, then decreased and finally increased again. The initially high rates may have been due to residual high activity since the experiment set up occurred at higher temperature. The final increase may have been due to fungal colonies which started to colonize the samples after a few weeks.

Addition of inocula that were already adapted to the fuel present shortened the lag phase and lead to a higher peak respiration for Syntroleum and fish biodiesel. Especially for the first two weeks, when mineralization rates were highest, biodegradation was faster with previously adapted microbes, except for diesel, where MPN showed lower microbial numbers in the inoculum which explained lower diesel degradation with a diesel-adapted inoculum. However, significant degradation was achieved with any type of inoculum under favorable conditions, i.e. at 20°C with nutrient addition. For the degradation of Syntroleum and diesel, cumulative CO_2 production after a period of 4 weeks was for all three types of inocula similar (± 15%). It can therefore be concluded that while inoculation with adapted microbes initially speeds up degradation, long term results are similar whether or not an adapted microbial consortium was initially present.

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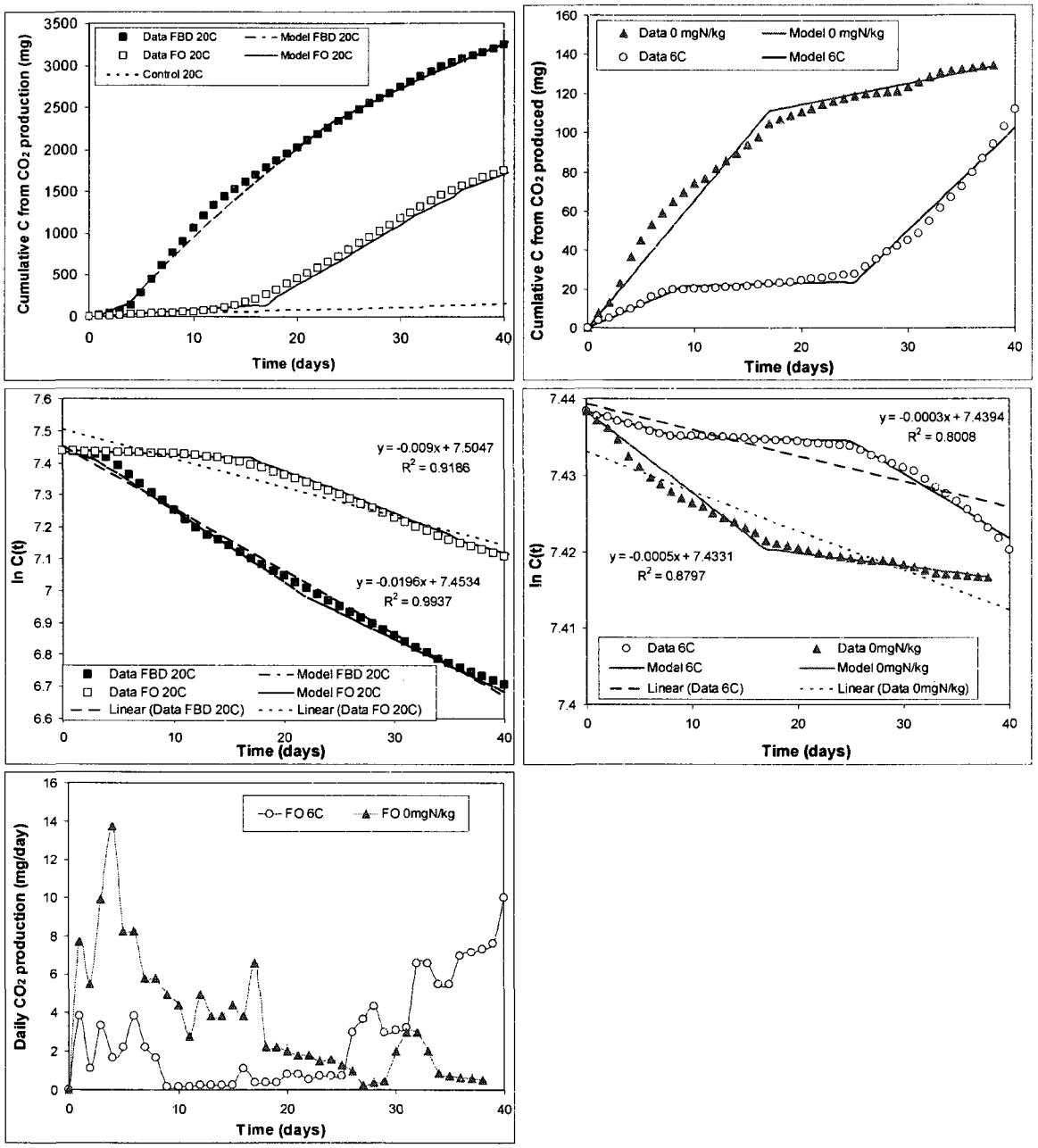


Figure 3.1 Degradation of fish oil and fish biodiesel over time for different conditions at a fuel concentration of 2000 mg/kg soil.

a) Cumulative respiration data for fish oil and fish biodiesel and 3-phase 1st order model with nonlinear parameter optimization at 20°C 300 mg N/kg,

b) Cumulative respiration data for fish oil and 3-phase 1st order model with nonlinear parameter optimization at 20°C 0 mg N/kg, versus 6°C and 300 mg N/kg, .

c) Linearized first-order plot for rate constant determination at 20°C and 300 mg N/kg.

d) Linearized first-order plot for rate constant determination at 20°C and 0 mg N/kg versus 6°C and 300 mg N/kg.

e) Daily respiration data at 20°C 0 mg N/kg and at 6°C 300 mg N/kg.

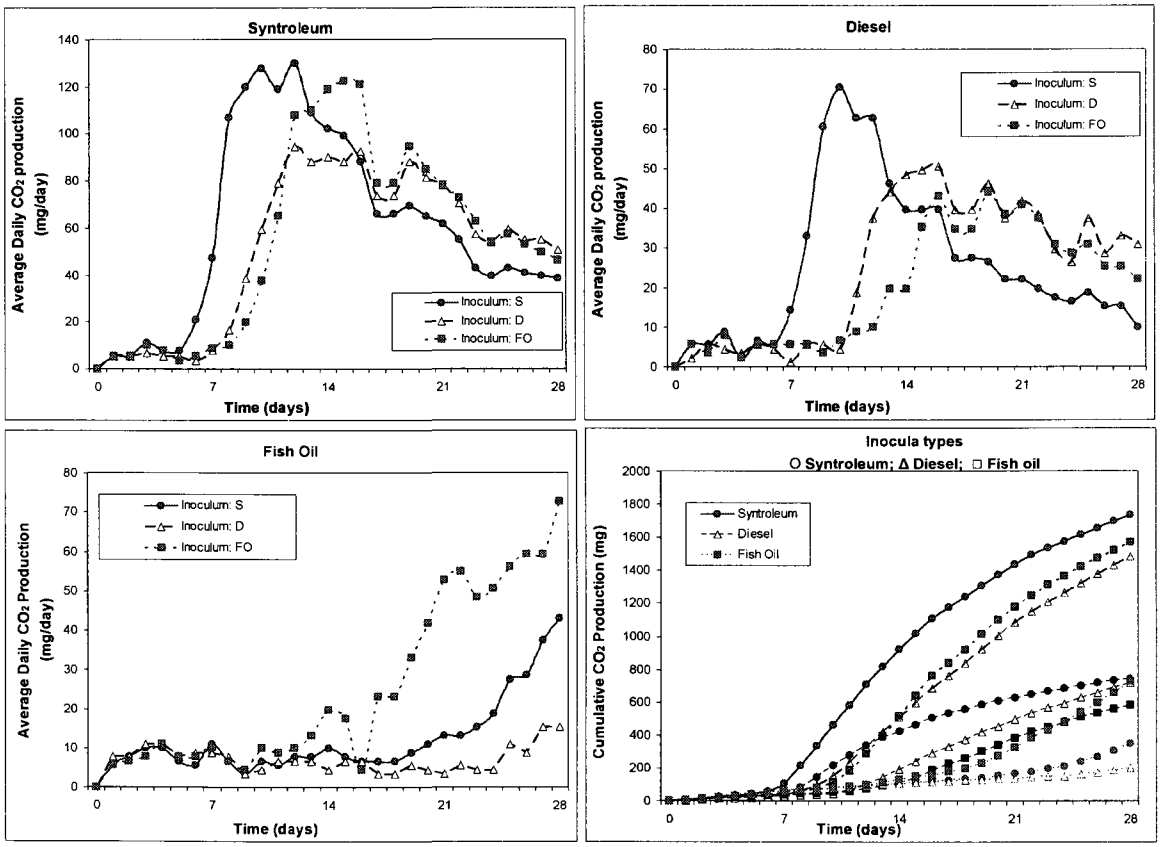


Figure 3.2 Mineralization of different fuels with Syntroleum, diesel or fish oil inocula. a) daily respiration for Syntroleum (S) substrate, b) daily respiration for diesel (D) substrate, c) daily respiration for fish oil (FO) substrate, d) cumulative

CO_2 production for all three fuel types. Conditions: 20°C; 2000 mg/kg of fuel; 300 mg N/kg.

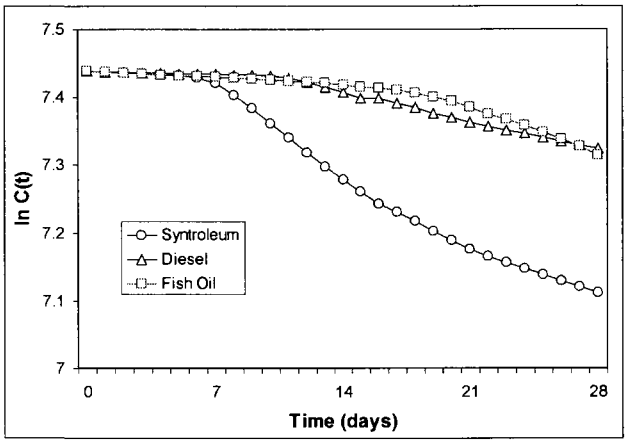


Figure 3.3 Linearized first-order plot for rate constant determination for the three fuel types with own inocula. Conditions: 20°C; 2000 mg/kg of fuel; 300 mg N/kg.

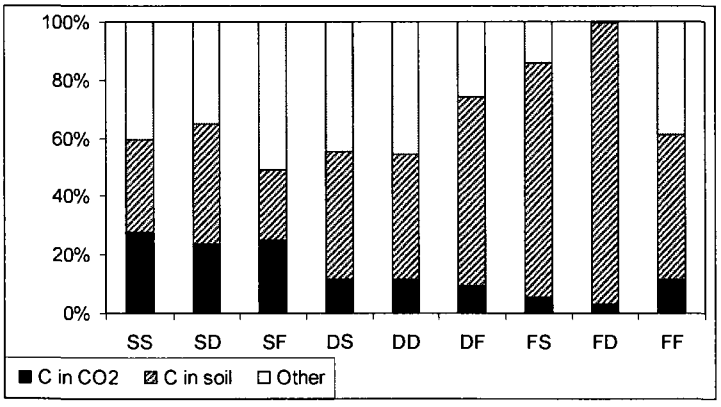


Figure 3.4 Carbon mass balance for the three fuel types after four weeks of degradation. Conditions: 20°C, > 10% gravimetric moisture content, 2000 mg fuel/kg dry soil. Abbreviations: first letter = fuel type added, second letter = type of inoculum, S=Syntroleum, D=diesel, F= raw fish oil.

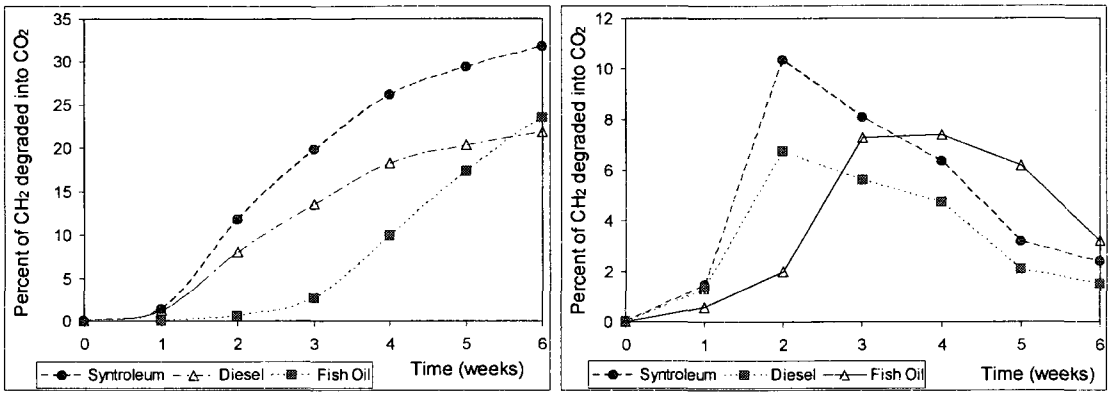


Figure 3.5 Mineralization of Syntroleum, diesel and fish oil over time with non-specific inoculum. a) Percentage of cumulative mineralization. b) Percentage of weekly mineralization. Conditions: 20°C, 2000 mg/kg of fuel, 300 mg N/kg.

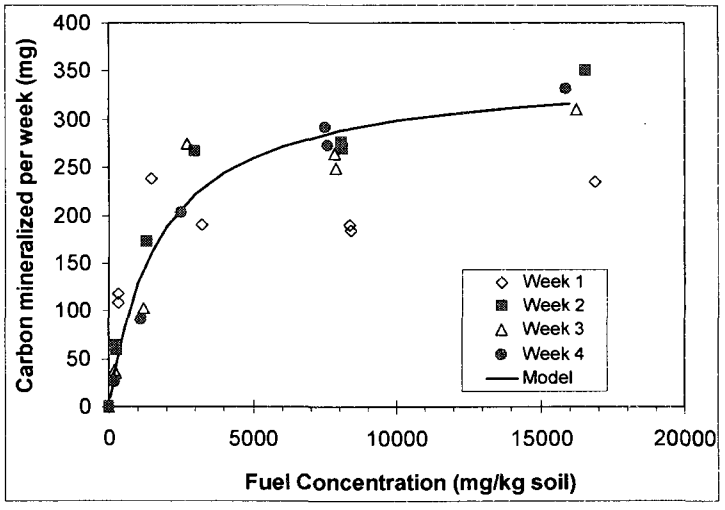
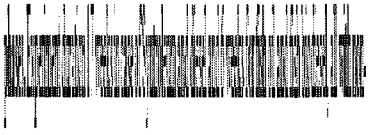


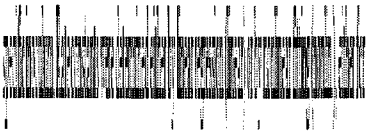
Figure 3.6 Cumulative Syntroleum mineralization per week as a function of remaining fuel concentration for different weeks. Conditions: initial fuel concentrations varying from 500 to 20,000 mg/kg dry soil, 20°C, 300 mg N/kg.

Table 3.1 Lengths of microbial growth phases for different environmental conditions and fuel types, determined based on first-order plot of *ln C* versus *t*. Data represent average of duplicates from 42, 56, or 120 day experiments with non-fuel-specific inoculum.

* two exponential phases with different rates; ' Experimental data based on 28 or 40 days; ~ initially high rate followed by lag phase

Fuel type	Conditions			Lag phase			Exponential phase		Stationary phase
	Temperature (°C)	Nutrient consumption (mg N/g fuel)	Fuel consumption (mg/g)	Model 1 (days)	Model 2 (days)	Model 3 (days)	Model 1 (days)	Model 2 (days)	Model 1 (days)
Jet	4	0	0	28	28	28	14	14	14
	4	1.5	1.5	28	28	28	14	14	14
	4	3	3	28	28	28	14	14	14
	4	6	6	28	28	28	14	14	14
	15	0	0	28	28	28	14	14	14
	15	1.5	1.5	28	28	28	14	14	14
	15	3	3	28	28	28	14	14	14
	15	6	6	28	28	28	14	14	14
	25	0	0	28	28	28	14	14	14
	25	1.5	1.5	28	28	28	14	14	14
	25	3	3	28	28	28	14	14	14
	25	6	6	28	28	28	14	14	14
Diesel	4	0	0	28	28	28	14	14	14
	4	1.5	1.5	28	28	28	14	14	14
	4	3	3	28	28	28	14	14	14
	4	6	6	28	28	28	14	14	14
	15	0	0	28	28	28	14	14	14
	15	1.5	1.5	28	28	28	14	14	14
	15	3	3	28	28	28	14	14	14
	15	6	6	28	28	28	14	14	14
	25	0	0	28	28	28	14	14	14
	25	1.5	1.5	28	28	28	14	14	14
	25	3	3	28	28	28	14	14	14
	25	6	6	28	28	28	14	14	14
Gasoline	4	0	0	28	28	28	14	14	14
	4	1.5	1.5	28	28	28	14	14	14
	4	3	3	28	28	28	14	14	14
	4	6	6	28	28	28	14	14	14
	15	0	0	28	28	28	14	14	14
	15	1.5	1.5	28	28	28	14	14	14
	15	3	3	28	28	28	14	14	14
	15	6	6	28	28	28	14	14	14
	25	0	0	28	28	28	14	14	14
	25	1.5	1.5	28	28	28	14	14	14
	25	3	3	28	28	28	14	14	14
	25	6	6	28	28	28	14	14	14
Biodiesel	4	0	0	28	28	28	14	14	14
	4	1.5	1.5	28	28	28	14	14	14
	4	3	3	28	28	28	14	14	14
	4	6	6	28	28	28	14	14	14
	15	0	0	28	28	28	14	14	14
	15	1.5	1.5	28	28	28	14	14	14
	15	3	3	28	28	28	14	14	14
	15	6	6	28	28	28	14	14	14
	25	0	0	28	28	28	14	14	14
	25	1.5	1.5	28	28	28	14	14	14
	25	3	3	28	28	28	14	14	14
	25	6	6	28	28	28	14	14	14





Growth Phases	Conditions			Lag phase		Exponential phase		Stationary and death phase		Overall	RMSE	RMSE
Food type	Temperature (°C)	Nitrogen concentration (mg/100 ml)	Food Concentration (cells/ml)	Linear	Nonlinear	Linear	Nonlinear	Linear	Nonlinear	Linear	Nonlinear	Overall Linear
Diesel	20	300	20000	0.000283	0.000277	0.000111	0.000111	0.000283	0.000282	0.000141	0.000087	0.000178
	20	300	40000	0.000283	0.000278	0.000111	0.000109	0.000283	0.000282	0.000141	0.000088	0.000177
	20	0	20000	0.000274	0.000274	0.000111	0.000109	0.000283	0.000283	0.000141	0.000086	0.000177
	4	300	20000	0.000283	0.000283	0.000111	0.000111	0.000283	0.000283	0.000141	0.000087	0.000178
Synechococcus	20	300	20000	0.000283	0.000282	0.000111	0.000111	0.000283	0.000283	0.000141	0.000087	0.000178
	20	300	40000	0.000277	0.000277	0.000111	0.000111	0.000283	0.000283	0.000141	0.000087	0.000178
	20	0	20000	0.000274	0.000274	0.000109	0.000109	0.000283	0.000283	0.000141	0.000086	0.000177
	4	300	20000	0.000283	0.000283	0.000111	0.000111	0.000283	0.000283	0.000141	0.000087	0.000178
Fresh Chl	20	300	20000	0.000277	0.000277	0.000109	0.000109	0.000283	0.000283	0.000141	0.000087	0.000178
	20	300	40000	0.000274	0.000274	0.000109	0.000109	0.000283	0.000283	0.000141	0.000087	0.000178
	20	0	20000	NA	NA	0.000109	0.000109	0.000283	0.000283	0.000141	0.000087	0.000178
	4°	300	20000	0.000283	0.000283	0.000109	0.000109	0.000283	0.000283	0.000141	0.000087	0.000178
Fresh Nondiesel	20	300	20000	0.000283	0.000283	0.000109	0.000109	0.000283	0.000283	0.000141	0.000087	0.000178
	20	300	40000	0.000274	0.000274	0.000109	0.000109	NA	NA	0.000141	0.000087	0.000178
	4°	300	20000	0.000274	0.000283	0.000109	0.000109	NA	NA	0.000141	0.000087	0.000178

Table 3.3 Microbial growth phases and degradation rate constants for Syntroleum, diesel and fish oil determined from the inocula study. Conditions: 20°C, 300 mgN/kg soil, 2000 mg fuel/kg soil. Experiment duration: 28 days. Abbreviations: first letter = fuel type added, second letter = type of inoculum, S=Syntroleum, D=diesel, F= fish oil; Values based on two phases, the lag phase and the exponential phase due to short length of experiment. Length of phases determined from best fit of 2-phase 1ST order model (method 4).

	Lag phase							Exponential and Stationary phases					Average			
	Time (days)		r^2	Rate constants (d^{-1})		RMSE		r^2	Rate constants (d^{-1})		RMSE		Rate constants		Overall Non linear	Overall linear
	Method 3	Method 4	Linear	Linear	Non lin.	Linear	Non lin.	Linear	Linear	Non lin.	Linear	Non lin.	(d^{-1})	r^2	RMSE	RMSE
SS	6	4.4	0.957	0.0016	0.0011	0.0013	0.0003	0.973	0.0148	0.0150	0.0316	0.0153	0.0142	0.977	0.0280	0.0141
SD	8	8.5	0.956	0.0010	0.0010	0.0006	0.0005	0.997	0.0140	0.0140	0.0048	0.0045	0.0114	0.957	0.0040	0.0038
SF	9	9.0	0.958	0.0013	0.0012	0.0011	0.0008	0.989	0.0157	0.0157	0.0134	0.0092	0.0125	0.947	0.0111	0.0076
DS	6	6.0	0.986	0.0009	0.0022	0.0009	0.0050	0.944	0.0056	0.0059	0.0189	0.0089	0.0056	0.965	0.0171	0.0082
DD	10	10.0	0.994	0.0007	0.0006	0.0003	0.0002	0.996	0.0067	0.0075	0.0063	0.0048	0.0051	0.936	0.0050	0.0039
DF	12	12.4	0.985	0.0009	0.0008	0.0006	0.0004	0.996	0.0059	0.0059	0.0076	0.0018	0.0039	0.912	0.0058	0.0014
FS	18	19.0	0.997	0.0012	0.0012	0.0004	0.0007	0.944	0.0037	0.0035	0.0157	0.0028	0.0017	0.906	0.0094	0.0017
FD	24	25.0	0.981	0.0010	0.0011	0.0022	0.0014	0.906	0.0022	0.0019	0.0236	0.0006	0.0010	0.983	0.0091	0.0014
FF	15	16.5	0.976	0.0015	0.0011	0.0018	0.0034	0.982	0.0084	0.0088	0.0084	0.0028	0.0040	0.843	0.0059	0.0032
Average			0.977			0.0010	0.0014	0.970			0.0145	0.0056		0.936	0.0106	0.0050

4 Influence of constant and fluctuating temperature on biodegradation rates of fish biodiesel blends in contaminating Alaskan sand[1]

4.1 ABSTRACT

Biodiesel is compared with diesel fuel a more environmentally friendly non-petroleum based hydrocarbon fuel made from renewable sources, such as animal fat. Biodiesel as a renewable energy source became a focus of interest in today's economy during the last few decades. Alaska's fishing industry generates large quantities of fish waste that after conversion to biodiesel it could lessen diesel fuel dependency in some remote Alaskan areas. In this study, fish–oil–based biodiesel and its blends with heating diesel fuel were investigated. Interior Alaskan, largely homogenous sand was spiked with heating diesel fuel as a control substance, processed fish biodiesel (B100), and its 5, 20, and 50% biodiesel blends with diesel fuel (B5, B20, and B50 respectively). The research was conducted with the main focus on biodiesel and biodiesel blend degradability in the subarctic environment and also the temperature effects on the degradation rates. The analysis also investigated the adaptation period (lag times) and different growth phases (exponential and stationary/death) of the microorganisms for different fuel types and

[1]Horel, A. and S. Schiewer. 2009. Influence of constant and fluctuating temperature on biodegradation rates of fish biodiesel blends in contaminating Alaskan sand. Prepared for submission in Bioresource Technology.

temperatures. The respiration rate (CO_2 production) was measured as an indicator of microbial activity and mineralization of contaminants. At the end of the experiment the remaining hydrocarbon concentration in the soil was analyzed by Gas Chromatography/Mass Spectrometry. For determining the nutrient depletion from soil High Pressure Liquid Chromatography has been used.

During a 28 day experiment, degradation rates increased with increasing percentage of biodiesel in the fuel. Pure fish biodiesel in showed the highest degradation rate with average degradation rate constants being 2–3 times higher than for pure diesel, which had the lowest rate. Rate constants varied between $k_{aveF} = 0.0280\ d^{-1}$ and $k_{aveD} = 0.0084\ d^{-1}$ at 20°C, $k_{aveF} = 0.0110\ d^{-1}$ and $k_{aveD} = 0.0037\ d^{-1}$ at around 12°C, and between $k_{aveF} = 0.0004\ d^{-1}$ and $k_{aveD} = 0.0002\ d^{-1}$ at 6°C. Temperature was an important factor. At 6°C, degradation was very slow and the lag phase persisted throughout the experimental duration, while lag phases were around 10 days at an average temperature of 12°C and 5 days or less at 20°C. During 28 days, mineralization reached over 50% for pure fish biodiesel at 20°C, while it was lower than 2% for all the fuels at 6°C. The Arrhenius plot was applied to determine the activation energy and pre-exponential factor. Based on CO_2 production and GC/MS analysis of remaining hydrocarbons, 50–85% of the initially present fuel could be accounted for. Part of the unaccounted quantity could have been incorporated into microbial biomass. This is supported by the finding that nitrogen depletion was highest in those cultures with the most active hydrocarbon degradation.

Even a low amount (5%) of biodiesel addition to the diesel fuel accelerated the biodegradation process significantly, more than expected. Routine addition of some biodiesel percentage to diesel fuel could help improve biodegradation after fuel spills and lessen effects on the environment.

Keywords: Biodegradation, biodiesel, biodiesel blend, degradation rates.

4.2 INTRODUCTION

Biodegradation of petroleum and other hydrocarbons by naturally occurring microorganisms is one of the cheapest remediation options especially in the Arctic and subarctic environment, where the limited infrastructure and difficulties in transportation can be limiting factor for a fast response. Natural attenuation as a remediation process can be one of the most feasible applications; however it is a relatively slow process and very time–limited due to short summer months in Alaska. Alternative fuel sources, such as vegetable or animal fat–based biodiesel, became of interest during the last few decades as a component of lessening dependency on petroleum products and to lower environmental pollution due to cleaner burning fuel. The research studied the degradation of heating diesel, pure fish biodiesel, and biodiesel blends with diesel fuel at different volume percent by naturally occurring microorganisms in Interior Alaskan contaminated sand. It had been reported by several studies that after successful laboratory experiments were conducted on biodegradation of hydrocarbon contamination, in the field minimal hydrocarbon mineralization was observed. Laboratory cultivated microbial cultures might

have significantly lower degradation effectiveness when applied in Alaskan soil, where the microorganisms not only compete for the substrate with indigenous species (Leahy and Colwell 1990), but also have to adopt to the new environment that is different from the laboratory bench-scale conditions. However, when microbial culture is cultivated with microorganisms from Alaskan soil, the lab results can be more applicable to the field. Communities previously exposed to hydrocarbons are already adapted to the carbon source and can respond to the presence of contaminant in the field much faster, even within hours (Atlas and Bartha 1998). As earlier mentioned, most studies find that biodegradation in laboratory experiments has much higher rates compared with field studies, however some research found opposite results, where investigating in unsaturated sandy soils the microbial degradation of hydrocarbons, especially the volatile compounds, in laboratory studies in cold environments are much lower compared with field studies (Hohener, Dakhel et al. 2006).

Due to a short summer period and extremely cold and long winters with lack of daylight, Alaska has high energy needs. Biodiesel is a source of renewable energy that can easily be stored and is not as affected by variable weather as solar and wind energy. According to Steigers et al., Alaska generated 8 million gallons of fish oil per year as a byproduct of the commercial fish industry (Steigers, Seshadri et al. 2004). The Alaska Energy Authority estimates that 13 million gallons of fish oil per year can be processed into biodiesel (AEA 2007). In general, raw fish oil cannot be called biodiesel without further processing. In this study only the already processed fish biodiesel was investigated. Fuel

for this study was created as follows: bulk fish oil was purchased from processors working in the North Pacific just off the Alaskan coast in 24,000 liter lots (one full ISO shipping container). This raw fish oil was shipped to Hawaii for conversion to biodiesel, through the transesterification process by a commercial biodiesel plant and returned to Alaska (Schmid, 2008, Pers. Comm.)(Witmer and Schmid 2008). Oxidized fish oil biodiesel samples were rehabilitated by adding 400 ppm by volume of the antioxidant, ethoxyquin and passing the fuel through a bleaching column of clay to remove fats and oxidized components (Schmid, 2008, Pers. Comm.). In rural Alaskan communities the heating diesel fuel price is much higher than the national or even Alaskan average gas prices. The use of locally processed fish biodiesel as a heating fuel by mixing with high sulfur diesel fuel would be feasible. In 2005 fish biodiesel had been tested to power generators and trucks in the Denali and Katmai National Parks as a part of the Alaska Biodiesel Demonstration project (FNS 2005). Pure fish biodiesel is generally believed to be non toxic and highly biodegradable; however since the effects of biodiesel blends on the environment and its actual biodegradability in cold climates are currently unknown, the objective of the current research was to investigate degradation of biodiesel blends at different temperatures.

4.3 MATERIALS AND METHODS

4.3.1 Methods

Small–scale experiments were conducted to study the rate of hydrocarbon degradation over time for 5 types of fuels (heating diesel fuel, processed fish biodiesel and three fish biodiesel blends with heating diesel fuel) and three temperatures (6°C, varying temperature around 12°C, and 20°C). Moisture content, nutrient dosage and soil type were the same for all experiments. Fish biodiesel blends were prepared on the volume percent based method (ASTM D 6751 – 08, 2008).

For each experiment, 1 kg of soil (sand) was placed in an airtight 2.5 L container. Quantified amounts (2000 mg/kg dry $soil^{-1}$) of the chosen contaminant, processed fish biodiesel, heating diesel fuel, blends of both containing 5, 20, or 50% biodiesel, were added to the surface of previously uncontaminated soil. Additionally, a small amount of soil with known existing microbial cultures from previous experiments that were already adapted to diesel fuel hydrocarbons was added to provide an inoculum of microbes and enhance degradation. Fertilizer of the type 20–20–20 ($N–P_2O_5–K_2O$, where the total nitrogen ingredients were: 20% ammoniacal, 30% nitrate, and 50% urea nitrogen) was dissolved in 10 ml of water with a final concentration of 30 mg N/ml of solution, and was sprinkled over the soil surface, achieving nutrient dosages of 300 mg N/kg dry soil. The soil was not mixed after or during these additions to better represent natural conditions after a spill on the soil surface. The samples were incubated either at a constant 6°C or 20°C, or a fluctuating temperature ranging between 6°C or 20°C. For most experiments,

some water was added, maintaining a gravimetric moisture content fluctuating between 4 and 6%.

Data were collected during a 4 weeks period. Carbon dioxide is the main product generated when a substrate is completely mineralized in aerobic degradation processes. Therefore, the respiration rate, that is, the rate of CO_2 production, was measured as an indicator for microbial activity. Typically, the container was opened once daily for re-aeration and to measure the evolved CO_2, which had been captured in 20 ml of 1 N NaOH solution (Page, Miller et al. 1982). During the daily analysis of the small-scale experiment, CO_2 measurements were taken by using a method and formula developed by Stotzky (Page, Miller et al. 1982). The carbonate formed was precipitated with a 0.3 N $BaCl_2$ solution.

To relate carbon dioxide production quantitatively to the degradation of hydrocarbons, the following stoichiometric equation for CO_2 production was used, assuming a generalized C:H ratio of CH_2 for diesel:

$$2\ CH_2 + 3\ O_2 \rightarrow 2\ CO_2 + 2\ H_2O. \qquad \text{eq. 4.1}$$

This stoichiometric equation applies only to mineralization of the fuel. The amount of CO_2 measured as a result of microbial consumption of hydrocarbons might be different from the actual hydrocarbon decrease in the soil. Use of this equation to estimate the amount of fuel degraded based on the amount of CO_2 produced can lead to over- or underestimations. The amount of fuel degraded may be underestimated based on CO_2

measurements, since not all substrate is completely mineralized, some being incompletely degraded or used for the production of new biomass. On the other hand, the total fuel degraded may be overestimated if some CO_2 is produced from other carbon compounds originally present in the soil. That scenario is unlikely in the present study, however, since soils with low organic content were used. The fraction of total carbon averaged only 0.165. The predominant importance of the added fuel compared to other possible substrates was confirmed by the use of controls without fuel addition. A baseline was developed based on uncontaminated soil samples for comparison with the calculated CO_2 production of the degradation experiments.

Fluctuating air temperature data was recording at least once a day. A separate thermocouple experiment was also carried out at different soil moisture levels to correlate between air and soil temperature data, where the investigated soil samples used were the same as in actual experimental setups. Thermocouples were placed at two different depths, one directly below the top of the soil (app. 1 cm) and the next approximately four cm below the soil surface. Three different soil moisture contents were used, one fully saturated soil, one with 4.8% GWC (same as in actual experiments), and one with dry soil. Thermocouple data were recorded on a data logger (DX10).

First-order degradation rates were determined by using the integral method based on the calculated amount of substrate still available according to respiration data,

$$\ln C_t = \ln C_0 - k\,t \qquad \text{eq. 4.2}$$

where C_t is the remaining contaminant concentration at any given time t; C_0 is the initial contaminant concentration; and k is the rate constant. According to this equation, in a plot of ln C_t versus time, the data points should fall on a straight line with the slope $-k$ if first-order kinetics applies, that is, if the rate of degradation is proportional to the remaining amount of contaminant at any given time. The rate constant corresponds to the slope of a regression line through the data. Hydrocarbon mineralization rate constants and time periods for different growth phases were optimized by minimization of the root mean square error (RMSE).

The effect of temperature on reaction rates was investigated by using Arrhenius' equation for known rate constants at different temperatures,

$$k = A_f \exp(-E_a / RT) \qquad or$$

$$\ln(k) = (-E_a / R)*(1/T) + \ln(A_f) \qquad \text{eq. 4.3}$$

where k is the temperature dependant rate constant; A_f is the pre-exponential factor determined from the Arrhenius plot; E_a is the activation energy also determined from the Arrhenius plot; R is the gas constant (8.314 J/mol); and T is the temperature in Kelvin.

To see if the soil temperature at different depths can be modeled and consequently predicted, and the following equation been used (Cengel 2007) with the assumption of heat flux is being constant:

$$\frac{T(x,t)-T_{air}}{T_{soil}-T_{air}} = erfc\left(\frac{x}{2\sqrt{\alpha t}}\right) \qquad \text{eq. 4.4}$$

where α represents the thermal diffusivity (m^2/s), T is the temperature (K), x is the distance (m), and t is the time (seconds). T_{soil} is a measured soil temperature at known distance and $T(x,t)$ is the calculated soil temperature at any given x distance from soil surface and.

To calculate the characteristic times it takes the soil to correspond to the air temperature, the following equation was used:

$$CT \approx \frac{L^2}{\alpha} \qquad \text{eq. 4.5}$$

where CT represents the characteristic time (seconds), L is the length approximated as half of the soil column, which is the vertical distance from the top of soil surface, and α is the thermal diffusivity (m^2/s) and calculated from

$$\alpha = \frac{k}{(\rho * c_p)} \qquad \text{eq. 4.6}$$

where k represents the thermal conductivity of the soil (W/mK), ρ is the density of the soil (kg/m^3) and c_p is the specific heat (J/kg K). The value of α was fitted to the data.

To evaluate the relationship between substrate use and CO_2 production, gas chromatography/mass spectrometry (GC/MS) analysis was performed (Agilent Technologies 6890N Network GC System coupled to a 5873 mass selective detector with

column parameters: 30 m by 250 μm by 0.25 μm) and compared to respiration data. For this purpose, the diesel range organics (DRO) determination was conducted using a modified method that was developed based on the AK 102 (ADEC 2002) and EPA 8270 (semi volatile organics by GC/MS) methods (EPA 1996). The GC/MS DRO method used a spitless injection with helium carrier gas (pressure: 6.895 kPa; flow: 0.5 ml/min; average velocity: 26 cm/sec). The oven temperature started from 40° C till 320° C for the duration of 52.33 runtime (87.22 minutes) to ensure no carry–over contamination between different samples. Surrogate was used to evaluate the extraction efficiency and internal standards were used to qualify instrument reliability after being in use for a longer time period. Recovery data analysis was performed for determination of the actual hydrocarbon recovery value by the gas chromatography method used. For this purpose, a known amount of fuel was added to the soil and extracted immediately. The highest recovered hydrocarbon amount for each fuel type (between 93 and 104%) for was taken as a reference value of 100% for the GC/MS data analysis instead of the theoretical value (calculated by using a generalized stoichiometric equation).

4.3.2 Soil characteristics

The soil used in the experiments was not sterilized and thus contained some naturally occurring microbial cultures that survive extreme conditions (–50° C to 30° C). The purpose for using unsterilized soil was to further simulate natural degradation processes. General soil analysis was performed using a Dionex DX 500 ion chromatograph with PeakNet software for nitrite and nitrate analysis and the ASTM standard for sieving.

Analysis for total nitrogen and total carbon concentration was done by using an elemental combustion system (Costech Instruments). The average percent of total nitrogen in the soil was 0.0054% and the fraction of total carbon was 0.165%.

The pH of the soil was analyzed before the experiments, using a Mettler Toledo pH meter at 21.8°C. The soil pH value was between 7.07 and 7.25, which is optimal for hydrocarbon degradation (Saadoun and Al-Ghzawi 2005). The original water content before the start of the experiments was negligible, less than 1%. The bulk density and porosity of the soil was determined by using the ASTM standard. The soil particles were ranging mainly between coarse to very fine sand with less than 0.3% of clayey material. The average bulk density was 1.4–1.6 g/cm^3. The average porosity of the sand was between 39.7 and 49.3% depending on the amount of clay present in the soil. Additional measurement of hydraulic conductivity averaged $2.6*10^{-2}$ cm/s as measured using a portable air permeameter (Tiny Perm II).

4.4 RESULTS

4.4.1 Effect of blend percentage on degradation rates

The daily and cumulative CO_2 evolution (mg/day and mg respectively) from the different fuel types are shown in Figure 1. In general, the lowest respiration rates were observed in the diesel fuel contaminated soil samples (k_{ave} = 0.0083 d^{-1} at 20°C) and the highest respiration when pure fish biodiesel was contaminant source. The highest difference between fuel types could be noted at 20°C. Figure 1 shows a trend where a higher

biodiesel percentage lead to faster mineralization of the contaminant. At 20°C and for fluctuating temperature, 20% biodiesel addition to the diesel fuel almost doubled cumulative respiration, while B100 had almost three times higher CO_2 production than diesel fuel (Figures 2b and d). Peterson and Möller (1998) investigated plant based biodiesel blend biodegradability and similarly concluded that addition of biodiesel to diesel fuel enhances the mineralization process of the contaminant by microbial activities (Zhang, Peterson et al. 1998). Biodiesel fuel is less toxic to the microorganisms in the soil also more easily metabolized than diesel due to its natural compounds consisting of pure fatty acids that are hydrocarbon chains and being recognized and used as a carbon source faster by microbial enzymes (Zhang, Peterson et al. 1998). The increase in degradation with increasing biodiesel percentage observed here was more pronounced than would have been expected by linear interpolation between diesel and biodiesel performance, which mean that addition of biodiesel to the diesel fuel enhances biodegradation. Figure 1 b, d, and f shows expected cumulative degradation of blends based on linear interpolation. The observed blend degradation clearly exceeds expectations. This could be explained by enhanced microbial growth in the presence of biodiesel, aiding degradation of conventional diesel.

When respiration peaked after about one week, a blend with 5% of biodiesel increased the daily respiration peak by a factor of 1.6 and a further increase of the blend percent, from 5 to 20% by volume, increased the respiration peak an additional 1.4 times at 20°C (Figure 1a). The daily highest respiration peaks were 83, 125, 179, 179 an 188 mg CO_2/d

for diesel, B5, B20, B50 and B100 respectively at 20°C. The peaks in daily respiration were similar between B20, B50, and B100. The subsequent decline in daily respiration was slowest for pure fish biodiesel while respiration declined faster as the biodiesel percentage in the fuel decreased. With time, the respiration rates of most fuels at 20°C became similar. Two weeks after starting the experiment, diesel and B5 fuels already had very similar daily respiration rates (≈ 40 mg CO_2/day) while B20 continued for an additional week at higher rates than diesel.

4.4.2 Fraction of hydrocarbon mineralized for different fuel types and temperatures

Since blanks without any fuel addition showed very low cumulative respiration of 158 mg CO_2 produced in 40 days at 20°C, it can be assumed that all CO_2 produced originated from the fuel source added to the soil. Figure 2 shows the percentage of different fuels that was mineralized within 28 days at each of the three temperatures. The fraction of hydrocarbons mineralized after 4 weeks was the greatest for B100 with an average percentage of 51.6% of the original contaminant converted into CO_2 and water at 20°C (Figure 2). Compared with previous studies this amount is very significant, as the original unprocessed fish oil showed only 22–26% of mineralization after 6 weeks (Horel and Schiewer 2007). The chemical differences between the fuel types before and after the transesterification process make the fish biodiesel more biodegradable than pure fish oil.

As the amount of fish biodiesel in the fuel decreases, the amount of mineralization decreases as well, with diesel averaging 18.5% after 4 weeks (Figure 2), which is similar

as determined for number 2 diesel fuel, which was on average 23.1% mineralized after 4 weeks under comparable conditions (Chapter 2). For 20°C, the relationship between blend percent and cumulative C (mg) evolved as CO_2 can be best described by a linear equation of $Y = 0.31X + 0.23$ with $r^2 = 0.995$, where X is the percentage of pure fish biodiesel in the fuel and Y is the fraction of C evolved from CO_2 (Figure 2).

At fluctuating air temperature around 12°C, a similar trend was noted of increasing mineralization with increasing biodiesel percentage, however the mineralization percentage was about half of that observed at 20°C. The factor by which mineralization increased due to a raise in temperature from around 12°C to 20°C varied between 1.65 for B50 and 2.11 for B5. At the lowest temperature, 6°C, minimal mineralization occurred during the 4 weeks study; less than 2% of the total contaminant was converted into CO_2 and water for any of the fuel types. However, it has been shown in longer term studies that even though respiration was very low for the first 20 days, after a period of 4 months the mineralization at 6°C reached 2/3 of that observed at 20°C (Chapter 2).

In a carbon mass balance, carbon dioxide plus carbon remaining in the soil (per GC/MS analysis) added up to 45–85% of the initially present carbon. The remaining 15–55% not accounted for can be due to volatilization, incomplete degradation, or incorporation in new microbial biomass. The effect of volatilization and fungal degradation of volatile compounds is discussed in a further paper by the present authors (Chapter 5). Consumption of nutrients can serve as evidence for production of new biomass.

4.4.3 Nutrient availability

Nutrients in the soil play an important role in the biodegradation process. Nutrient deficiency or excess in the soil can inhibit degradation processes (Ferguson, Franzmann et al. 2003a; Walworth, Woolard et al. 2003; Walworth, Pond et al. 2007). The original amount of nutrient as nitrogen per kg sand was adjusted to 300 mg N based on optimal nutrient levels determined by Niemeyer (2003) for microbial degradation of diesel fuel contaminated subarctic soil under similar environmental conditions (Niemeyer 2003). Previous research carried out by using the same soil type with varying nutrient addition also showed that 300 mg N/kg soil enhances the degradation process significantly (Chapter 2). At the end of the 28 days an analysis was carried out to determine the remaining amount of total nitrogen (nitrite and nitrate) in the soil by HPLC. The averaged results are shown in Figure 3. Comparing the 5 different fuel types, a fairly linear trend could be observed at high temperature; the lowest nutrient depletion occurred for diesel and with increasing fish biodiesel addition, nutrient depletion increased. The largest nutrient depletion of over 38% occurred for pure biodiesel. The fact that more nitrogen was depleted for those fuels that underwent higher microbial degradation, indicates that nutrient depletion appears to have been linked to microbial degradation of hydrocarbons. It is plausible that the depleted nitrogen was incorporated into microbial biomass. Based on the nutrient consumption of 11–38 %, shown in Figure 3, the theoretical amount of biomass produced according to an average biomass composition of $C_5H_{12}O_2N$ can be calculated as 8–29% according to eq. 4.6

$$\%\ \text{C converted to biomass} = \%\ \text{N used} * \frac{300\ mg\ N_{initial}}{1700\ mg\ C_{initial}} \frac{60\ mg\ C_{biomass}}{14\ mg\ N_{biomass}}. \qquad \text{eq. 4.6}$$

At lower temperatures the nutrient usage was less pronounced, no coherent conclusions could be drawn (Figure 3).

4.4.4 Effect of temperature on respiration rates

Interior Alaskan temperature has a large variation; indigenous species can survive the range from –50°C up to 30°C, however air temperatures between 1°C and 25°C could be considered as normal for the Interior–Alaskan summer, which is the most important time period for biodegradation where the largest contaminant mineralization can take place. Therefore, the following temperatures were investigated: constant 6°C, constant 20°C, and fluctuating temperature which averaged 12°C, where the temperature varied almost daily from 6°C to 20°C or vice versa as shown in Figure 4c. Several studies have found that increasing temperature increases respiration rates while investigating contaminated soils with cold–adapted microorganisms (Atlas and Bartha 1998; Walworth, Braddock et al. 2001; Ferguson, Franzmann et al. 2003b; Horel and Schiewer 2009) (Chapter 2).

Figures 4a and b show the daily and cumulative respiration data for three fuel types (diesel, B20, and B100) at different temperatures. When comparing the same fuel at different temperatures, at 20°C the cumulative respiration increased by 98% for diesel, 81% for B20, and 96% for B100 compared with 6°C (Figure 4b, Figure 2, and Table 1). B20 fuel had almost as much cumulative CO_2 production at around 12°C than diesel at

20°C. This means that addition of biodiesel can enhance degradation in a similar way as soil heating from around 12°C to 20°C, while being more cost efficient.

Figure 1c shows the daily respiration data for the fluctuating air temperature. Pronounced fluctuations of the mineralization rate were observed, which was not the case for the constant temperatures of 6°C or 20°C. The observed variability in respiration can be attributed to changing temperatures (Figure 4c).

As shown in Figure 4c, the temperature was, with some exceptions, manually changed every other day from high (~ 18°C) to low (~ 7°C) and alternating days from low to high. For B100 and B50 daily respiration values on those days where the temperature in the "T12" experiment peaked (Figure 1c, Figure 4a) were similar to those observed at a constant temperature of 20°C, with values of approximately 150 mg CO_2/day (Figure 1a, Figure 4a). When the temperature in the "T12" experiment reached the minimum of 6°C, the daily respiration rates were still relatively high compared with data at constant temperature of 6°C (Figures 1e and 4a), with values varying between 20 mg CO_2/day and 60 mg CO_2/day (Figures 1c and 4a) compared with less than 10 mg/g at 6°C (Figures 1e and 4a). Even from day 19 to day 21, when the temperature was kept low for three consecutive days, daily respiration rates stayed at similar levels as observed when the temperature dropped for only one day (higher than at 6°C) and increased sharply as soon the temperature increased as well (Figures 1c, 4a, and 4c). At day 25 and 26 the temperature was kept high for two days and the daily respiration rate increased on day 26,

reaching its maximum during the experiment for B50 and B100 (B100 = 156 mg CO_2/day).

To investigate the time delay between changing air temperature and subsequent change in soil temperature, thermocouple experiments were conducted with sensors placed either at the top portion or in the middle of a soil sample. Thermocouple data showed that for a decrease in air temperature from 20°C to 7°C over a period of two hours, approximately 8.5 hours are needed to reach soil temperature equilibrium at 7°C (Figure 5a). It was observed that higher soil moisture contents require longer time to equilibrate with air temperature (data not shown). In the actual experiment the gravimetric moisture content was 4.8%, which entails a relatively fast soil temperature response with near-equilibrium after 4 or 5 hours (Figures 5a and b). When the air temperature increased from 7°C to 16°C gradually over a period of one day, the soil temperature tracked the air temperature without delay (left side of Figure 5b), however when air temperature suddenly increased from 16°C to 20°C it took approximately 4 hours for the soil to reach equilibrium (right side of Figure 5b). Though small fluctuations of air temperature occurred due to the hourly control cycle (Figure 5a), soil temperatures showed very little variation. In conclusion, thermocouple experiments showed that the soil temperature lagged only a few hours behind air temperatures, so that the varying air temperatures shown in Figure 4c closely resemble the actual soil temperatures.

This means relatively (compared to 6°C data) high rates observed at temporarily low air temperatures (such as in the 12°C experiment from day 19 to 21) are not the result of soil temperatures remaining high, lagging behind air temperatures. Rather, the microbial consortium retains a high metabolic rate, lagging behind temperature changes, not yet having reduced their metabolic activity.

In conclusion, the respiration for the fluctuating temperature averaging 12°C, which could be considered the most realistic representation of a natural environment, showed that respiration rates were higher than expected based on extrapolation from that data at 20°C and 6°C. This is promising for bioremediation applications. It appears that the microbes can maintain an active state when temperatures drop for a few days.

Figures 1e and f show the daily and cumulative respiration data respectively for a constant low temperature of 6°C. The microbial activities were minimal and no end of the lag phase could be determined during the experimental duration. The cumulative respiration over the whole period of 28 days was similar to the averaged daily respiration at 20°C (Table 1). During the start of the experiment, the soil temperatures were higher than 6°C, which resulted in initially high respiration (Figure 2h). Even though soil temperatures should have dropped to 6°C in several hours (according to thermocouple data), a carry–over of high microbial activity apparently occurred as observed for days 19–21 in the fluctuating air temperature experiment.

The generally accepted rule of thumb is that the reaction rate doubles for each 12°C increase in temperature (Clark 1996). However this statement is overly generalized and not easily applicable for hydrocarbon removal rate constants. A larger increase in the reaction rates has been observed from 1°C to 11°C compared with a change from 11°C to 21°C (Walworth, Braddock et al. 2001). In the present study, the average rate constants approximately doubled from 12°C to 20°C, while from 6°C to 12°C they increased by approximately a factor of 20 (Table 2b).

The effect of temperature on reaction rates can be evaluated by fitting experimental data to the Arrhenius equation (3). In this research, three different temperatures were investigated and fitted to equation (3), with 4 to 6 data points for each plot, since some data were duplicated. The findings for all fuel types are shown in Figure 6 with the corresponding pre-exponential factors and activation energy. The data shows a moderate fit with r^2 values varying between 0.81 and 0.91; it was expected that the r^2 values would not be perfect due to the exceptionally small degradation rates at 6°C, where the microbial adaptation period was still ongoing (Table 2b). There was a much larger increase observed in degradation rate constants based on respiration data from 6°C to 12°C compared to the increase from 12°C to 20°C (Figures 4b and 6; Table 2b).

4.4.5 Microbial adaptation period and growth

The time between contamination of the soil and microbial adaptation to the changed environment is considered the lag phase. The shorter the lag phase, i.e. the sooner

biodegradation starts, the more complete degradation can be achieved during a relatively short time, such as Alaskan summer. For this reason it is important to shorten this phase and ensure long exponential and stationary phases when the microbial activities are highest. Most published studies use an intuitive approach for evaluating the length of the lag phase without further justification. There are several possible methods for defining the end of the lag phase, as described in detail in chapter 3:

1) When daily respiration exceeds a defined threshold value

2) When daily respiration increases significantly above previous days

3) When 1st order plot of *ln C* versus *t* shows sudden increase of slope

4) Such that a chosen mathematical model matches the data best

Since it is difficult to establish fixed criteria that are valid for different conditions for the first two methods, the 3rd and 4th method, which are more generally applicable, are used in this study. The third method focuses on changes in the specific rate per remaining substrate, as indicated by changes of slope in a linearized plot of ln C versus time. For example, in Figures 7a and b an increase of the slope can be observed at day 5 for 20°C, and at day 10 for 12°C, which means the length of the lag phase is 5 days or 10 days respectively.

To avoid subjective identification of a change in slope as done for the 3rd method, the fourth method applies a mathematical criterion: for a pre–determined number of phases, all model parameters (rate constants and length of all phases) are optimized at once by minimizing the root mean square error (RMSE) using Excel Solver function.

Figure 7a shows a first order plot with a regression curve for determining an overall rate constant from the slope of that line, as well as the results of a three–phase model with optimized rate constants and phase lengths for each of the phases (method 3 and 4). It is apparent that the three phase model fits the data significantly better than a model using one overall rate constant (straight regression lines). For the 20°C data the slope is initially small (lag phase), then increases around day 5 (start of exponential phase), and finally decreases around day 17 (start of stationary phase). The decline in daily respiration rates after the peak on day 6–8 (Figure 1a) does not indicate the death phase but can be explained by decreasing substrate concentrations while microbial activity (per substrate) remained high: the phase with the highest mineralization rate constant still continued till day 15–20 at 20°C.

Table 2 summarizes the rate constants and time periods of two growth phases. During the lag phase, rate constants were similar for all fuel types at a given temperature. In contrast to that, exponential phase rate constants differed by up to a factor of 4 between different fuel types. The lag phase durations were observed to be between 4 and 5 days at 20°C based on Figure 7a (criterion 3). When using the Excel Solver function to minimize error in the data (criterion 4), similar or shorter lag times were obtained. In the case of B20 the lag phase duration changed from 4 days for the 3rd method to 1.15 days for the 4th method and for B50 fuel the lag phase changed from 5 days to 2.73 days (Table 2).

At the lower temperature averaging 12°C, the adaptation period was longer, generally doubling compared with 20°C. Visual inspection of Figure 7b (method 3) yielded a lag time of 9 days, which corresponded well to the lag time 9–11 days obtained by RMSE minimization according to method 4 (Table 2).

Within the experimental duration of 28 days, the lag phase length for the lowest temperature, 6°C, could not be determined as no increase of rates over time was observed. An earlier study of the present authors had found lag times of over 20 days for conventional diesel and synthetic fuel (Chapter 3). Eriksson et al., (2001) noted however that at 7°C microbial degradation of hydrocarbons can start without a lag period while investigating arctic tundra soil (Eriksson, Ka et al. 2001). In the present study, the daily respiration data for the low temperature similarly showed high values during the first two days in the experiment compared with later days (Figure 1e). However, these values were still relatively small and no phases could be distinguished (Figure 7c). Furthermore, the relatively high activities for the first two days were likely a "carry–over" of high microbial activity since the soil was previously at room temperature as discussed in the section on temperature effects. It can be therefore concluded that the lag phase continued till the end of the present experiment. There were two (Table 2) or three main microbial growth phases (Figure 7) distinguishable at higher temperatures, the earlier–mentioned lag phase and also the exponential and/or stationary phase, which continued till the end of the experiment. Figure 7a shows the linearized first–order plot for three phases, distinguishing between exponential and stationary phases for diesel, B20, and B50 fuels

at 20°C and Figure 7b shows the general two phase model for the same fuels at 12°C. At higher temperature the 3–phase–model fits almost perfectly the data (Figure 7a), however when exponential and stationary phases are combined (Table 2a) the fit has a higher error (model for diesel fuel $RMSE_{2phase}$ = 0.0200 and $RMSE_{3phase}$ = 0.0027, data not shown). From the linearized first order plot, the degradation rate constants of exponential versus stationary phase at 20°C were quiet different; for example, for diesel k_{Dexp} = 0.0115 d^{-1} and k_{Dstat} = 0.0048 d^{-1}, and for B50 are k_{B50exp} = 0.0270 d^{-1} and $k_{B50stat}$ = 0.0145 d^{-1} (values not shown in Table 2), which becomes oversimplified when the two phases were combined as $k_{Dexp+stat}$ = 0.0081 d^{-1} and $k_{B50exp+stat}$ = 0.0222 d^{-1} (Table 2).

Figure 7d shows the measured degradation rate constants versus the expected degradation rate constants of the biodiesel blends at 20° C, based on liner interpolation between diesel and B100. The degradation rate constants observed by the experiments are considerably higher than the expected values, which means that addition of biodiesel to the diesel fuel enhances biodegradation and might result in a more complete.

4.5 CONCLUSIONS

Biodiesel and its blends with heating diesel fuel showed higher biodegradability than pure diesel fuel. Cumulative mineralization over the experimental duration of 28 days increased greatly when biodiesel was added to the diesel fuel, rising from 18.5% mineralization for diesel to 23.8% for B5 to 51.6% for B100 at 20°C. Hydrocarbons remaining in the soil and CO_2 produced accounted for 45–85% of the initially present

carbon. Some of the unaccounted carbon was converted into biomass, for which nutrient consumption was an indicator. The greatest nutrient usage was observed at 20°C for B100, where 115 mg N/kg soil was depleted by the end of the 28 day experiment, followed by B50 with 72 mg of N usage. This means up to 29% of the initially available carbon could have been converted to biomass. At lower temperatures, the nutrient usage was minimal and a conclusive trend could not be observed.

Biodegradation rates increased strongly with temperature and biodiesel percentage. At 20°C, the average rate constants varied between 0.008 d^{-1} for diesel and 0.03 d^{-1} for biodiesel. For temperature fluctuating around 12°C, the degradation rates were between 0.004 and 0.011d^{-1}. At 6°C, rate constants varied between 0.0002 d^{-1} and 0.0004 d^{-1}. Arrhenius plots did not show the expected linear relationship between overall rate constants. This was attributed to the fact that the lag phase continued till the end of the experiment at 6 °C. Thermocouple experiments showed that the soil temperature lagged a few hours behind changes in the air temperature. When the temperature was lowered, higher than expected rates were observed for several days.

Under optimal conditions, the microbial adaptation period was short, less than 5 days. However, when the conditions were less optimal, the lag phase considerably increased to about 10 days at 12°C and over 28 days at 6°C. Simultaneous optimization of rate constants and lengths for all phases, minimizing RMSE, lead to similar or smaller lag times as determined by inspection of a first order plot of the logarithm of the

concentration versus time. For the 6°C data, a model based on one overall rate constant could describe the data. For fluctuating temperature around 12°C and 20°C, two– or three–phase models fit the data much better since several growth phases could be distinguished.

4.6 ACKNOWLEDGEMENTS

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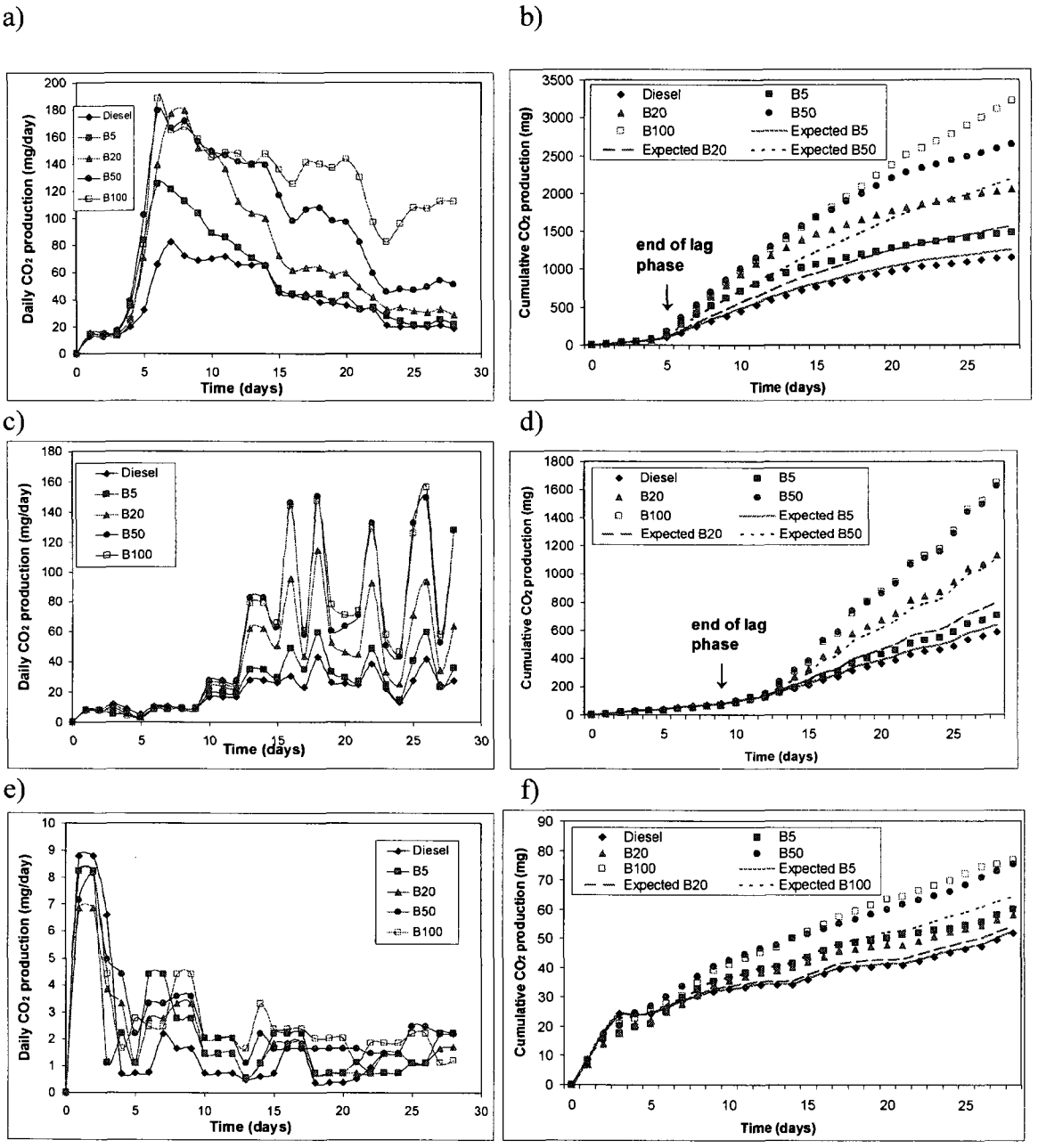


Figure 4.1 Daily CO_2 production (left) and cumulative mineralization (right) for B100, B50, B20, B5, and heating diesel fuel at different temperatures. Conditions: 2000 mg/kg of fuel; 300 mg N/kg; sand. Experimental duration: 28 days. Numbers

represent average values. a) and b) 20° C, c) and d) fluctuating temperature around 12°C, e) and f) 6°C.

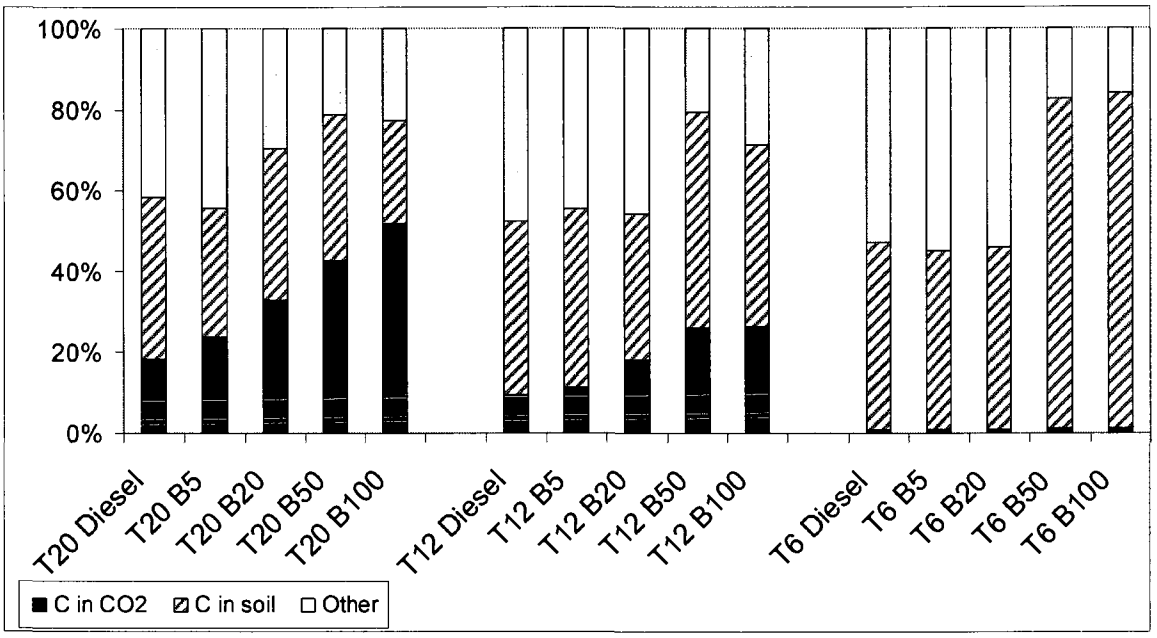


Figure 4.2 Percent carbon mass balance for different fuel types and temperatures. Conditions: 20°C (T20), fluctuating air temperature (T12), and 6°C (T6); 2000 mg/kg of diesel, B5, B20, B50, and pure fish biodiesel fuel, 300 mg N/kg, sand. Experimental duration: 28 days. Numbers represent average values with the assumption that all CO_2 originated from the fuel source.

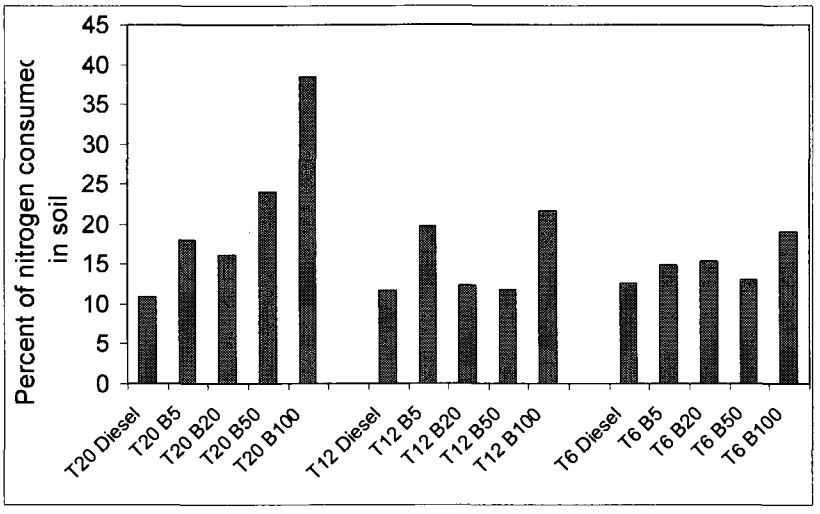


Figure 4.3 Nitrogen depletion from soil at different temperatures for the different fuel types. Conditions: 20°C (T20), fluctuating air temperature (T12), and 6°C (T6); 2000 mg/kg of heating diesel fuel, B5, B20, B50, and pure fish biodiesel (B100); 300 mg N/kg; sand. Experimental duration: 28 days. Numbers represent average values.

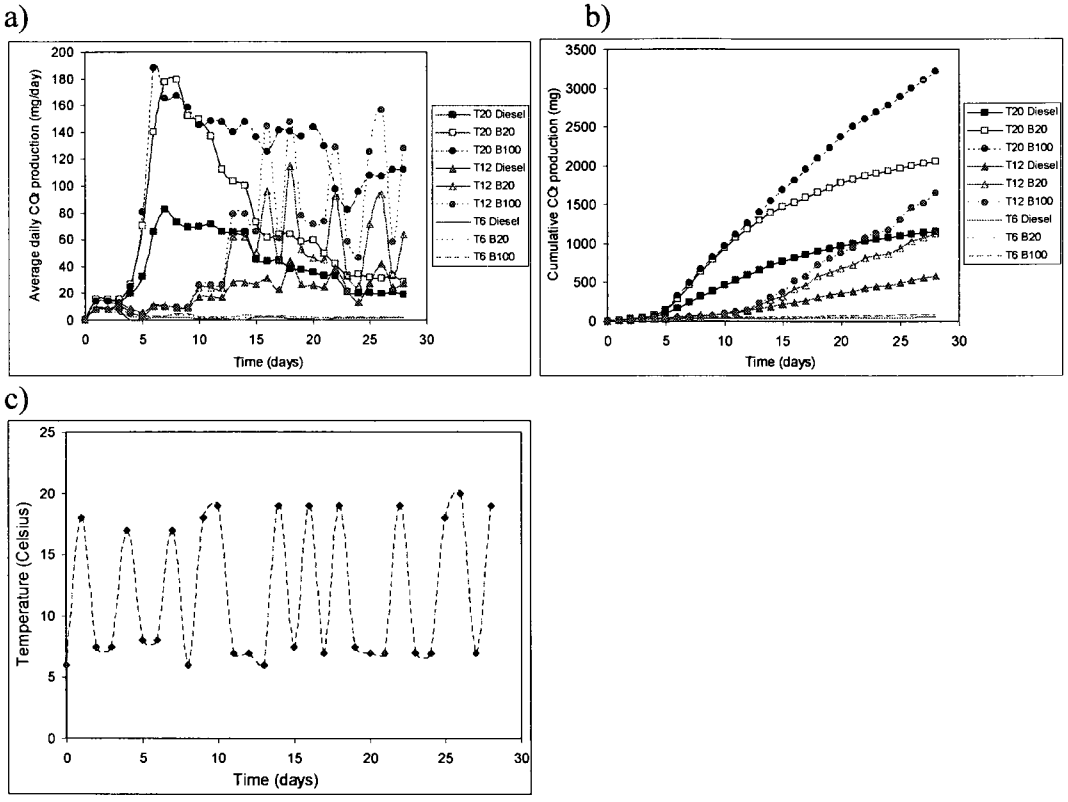


Figure 4.4 Mineralization of B100, B20, and heating diesel fuel at 20, around 12, and 6°C. Conditions: 2000 mg/kg of fuel; 300 mg N/kg; sand. Experimental duration: 28 days. Numbers represent average values. a) daily respiration b) cumulative respiration, c) actual variation for average temperature of 12°C.

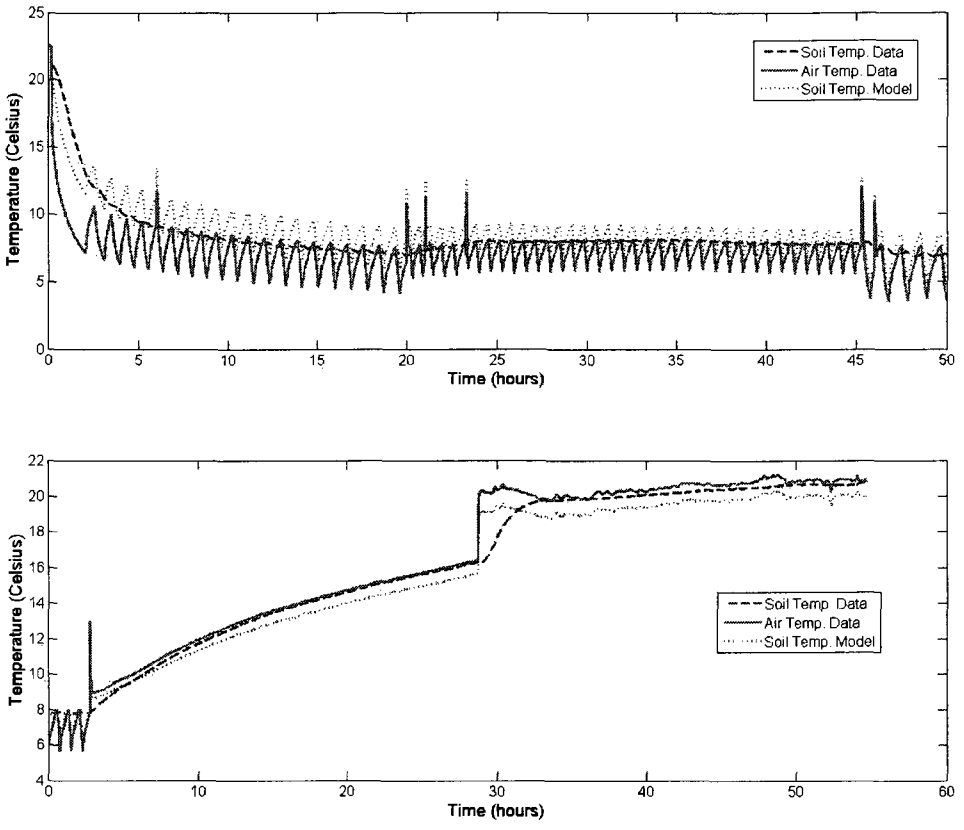
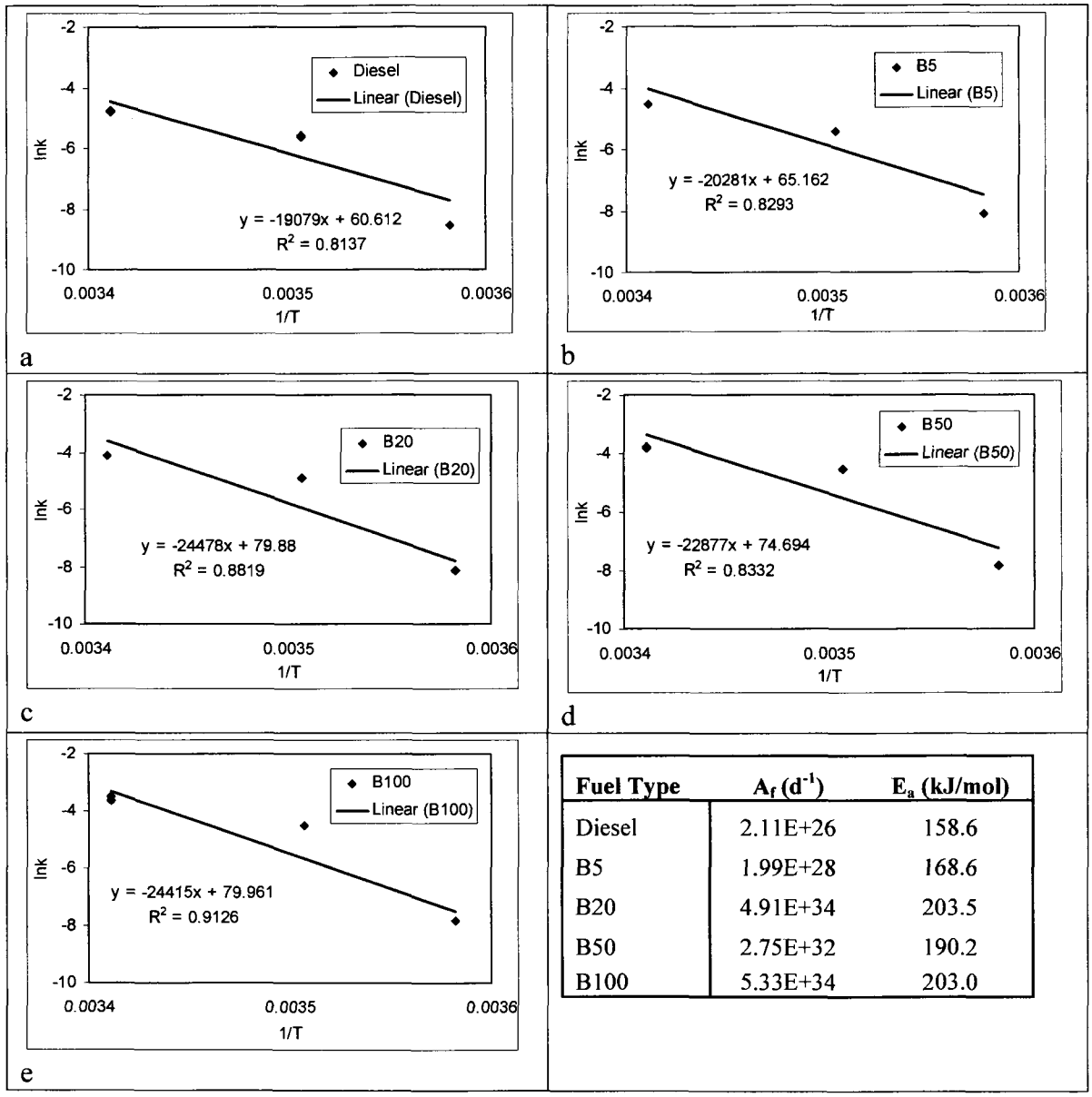


Figure 4.5 Soil temperature response to change in air temperature with sensors placed 4.5 cm below soil surface. a) decrease in air temperature and b) slow and sudden increase in air temperature. Condition: 4.8% GWC.



Fuel Type	A_f (d^{-1})	E_a (kJ/mol)
Diesel	2.11E+26	158.6
B5	1.99E+28	168.6
B20	4.91E+34	203.5
B50	2.75E+32	190.2
B100	5.33E+34	203.0

Figure 4.6 Arrhenius plot for determining Activation energy and pre-exponential factor for temperature–dependent overall rate constants and tabulated results of findings. Conditions: 20°C (293K), fluctuating air temperature (averaged 285K), and 6°C

(279K), 2000 mg/kg of **a)** heating diesel fuel, **b)** B5, **c)** B20, **d)** B50, and **e)** pure fish biodiesel (B100), 300 mg N/kg sand. Experimental duration: 28 days.

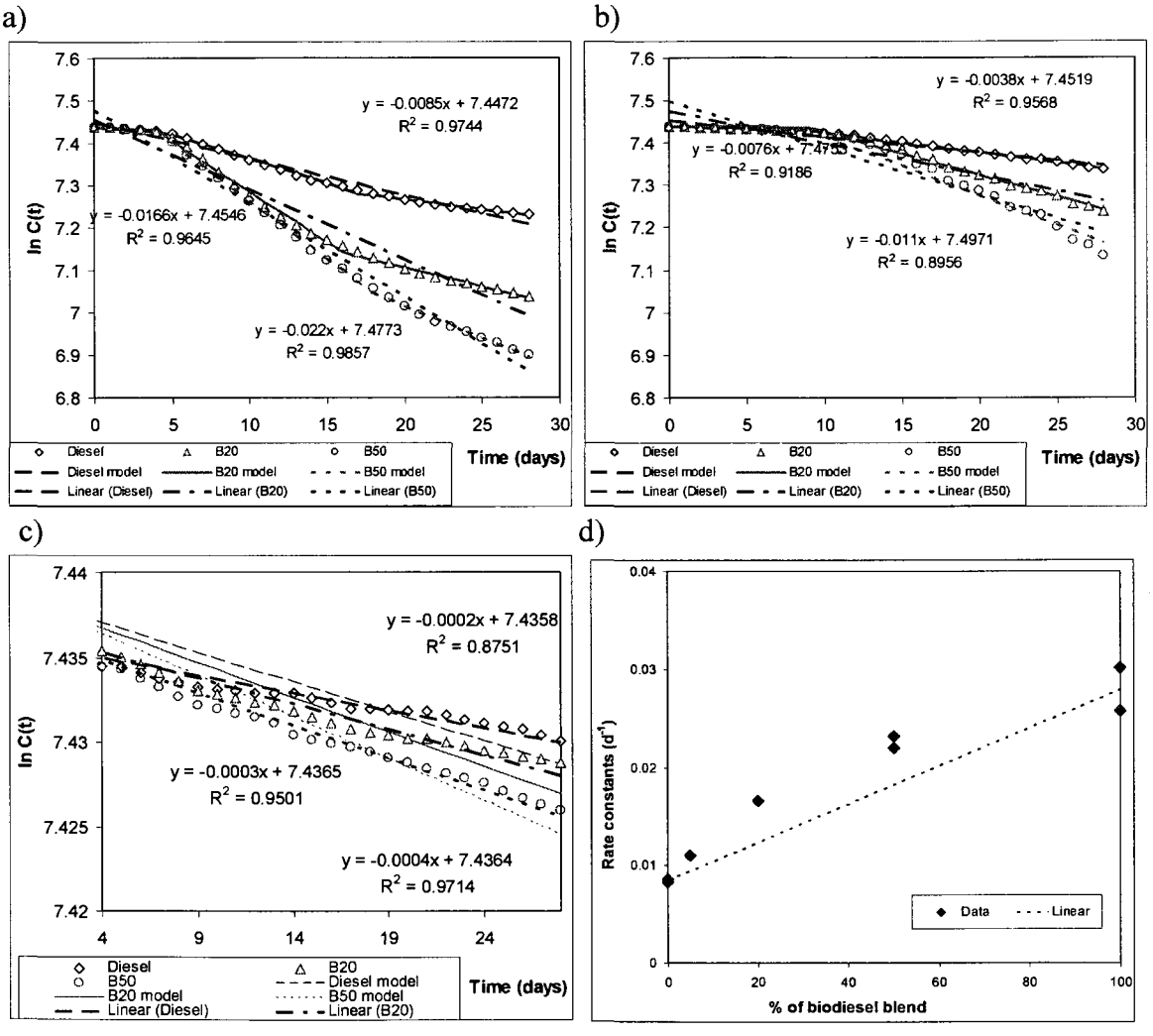
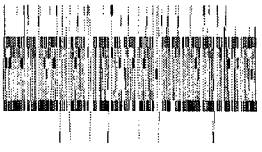


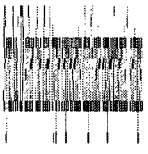
Figure 4.7 Linearized first-order plot for determination of the respiration rate constant using the integral method for diesel, B20 and B50. Data, overall linear correlation, and 1st order model with one or more phases for nonlinear parameter optimization with phase lengths according to method 3. Conditions: 2000 mg/kg of fuel; 300 mg N/kg; sand. a) 20°C with 3 phase model; b) 12°C with 2 phase model; c) 6°C

with 1 phase model; d) degradation rate constants measured for diesel, B5, B20, B50 and B100 versus expected performance of blends at 20°C.

Table 4.1 28–day–average daily and cumulative respiration data as well as model parameters (linear fitting) for the 5 different fuel types at different temperatures. Conditions: 20°C (T20), fluctuating air temperature (T12), and 6°C (T6); 2000 mg/kg of heating diesel fuel, B5, B20, B50, and pure fish biodiesel (B100); 300 mg N/kg; sand. Experimental duration: 28 days.

Fuel Types	Average daily respiration mg CO_2 /day	Cumulative respiration mg CO_2 / 28 days	Percent mineralized	Rate constants (k) d-1	r2	RMSE Non linear
T20 Diesel	41	1155	18	0.0084	0.976	0.0193
T20 B5	53	1482	24	0.0110	0.961	0.0291
T20 B20	73	2055	33	0.0166	0.965	0.0264
T20 B50	94	2637	42	0.0226	0.985	0.0206
T20 B100	115	3219	51	0.0280	0.986	0.0053
T12 Diesel	21	584	9.3	0.0037	0.959	0.0033
T12 B5	25	702	11	0.0045	0.938	0.0022
T12 B20	41	1134	18	0.0076	0.008	0.0035
T12 B50	58	1623	26	0.0109	0.892	0.0058
T12 B100	59	1647	26	0.0110	0.886	0.0063
T6 Diesel	1.9	52	0.82	0.0002	0.875	n/a
T6 B5	2.1	60	0.95	0.0003	0.925	n/a
T6 B20	2.1	58	0.92	0.0003	0.918	n/a
T6 B50	2.7	74	1.2	0.0004	0.949	n/a
T6 B100	2.7	77	1.2	0.0004	0.970	n/a





	Laryngitis						Experimental, Satisfactory, and Outside Guidance						Average	
	Length (days)		Patients monitored (#)		RMSE		Length	Patients monitored (#)		RMSE			RMSE	
Facility Type	?	Measure*	?	Measure*	Measure1	Measure2	(days)	Measure?	Measure*	Measure1	Measure2		Measure1	Measure2
T30Base	5	30	0.0025	0.0025	0.0011	0.0010	5-Level	0.0031	0.0077	0.0042	0.0025		0.0035	0.0018
T30BaseD	5	30	0.0025	0.0025	0.0014	0.0015	5-Level	0.0035	0.0104	0.0057	0.0029		0.0044	0.0022
T30I5	4	40	0.0025	0.0025	0.0012	0.0011	5-Level	0.0031	0.0075	0.0035	0.0020		0.0032	0.0016
T30I20	4	12	0.0025	0.0025	0.0009	0.0006	5-Level	0.0016	0.0058	0.0027	0.0017		0.0038	0.0011
T30I50	4	22	0.0025	0.0021	0.0020	0.0009	5-Level	0.0022	0.0028	0.0015	0.0012		0.0025	0.0011
T30I50D	5	27	0.0025	0.0044	0.0024	0.0009	5-Level	0.0024	0.0074	0.0036	0.0023		0.0031	0.0014
T30I100	4	41	0.0025	0.0024	0.0009	0.0005	5-Level	0.0021	0.0070	0.0017	0.0020		0.0024	0.0007
T30I100D	4	47	0.0025	0.0024	0.0007	0.0006	5-Level	0.0020	0.0051	0.0024	0.0007		0.0023	0.0010
T120Base	9	90	0.0014	0.0014	0.0002	0.0002	5-Level	0.0020	0.0055	0.0027	0.0014		0.0021	0.0010
T120BaseD	9	90	0.0015	0.0015	0.0002	0.0002	5-Level	0.0024	0.0062	0.0031	0.0014		0.0024	0.0010
T120I5	9	90	0.0014	0.0011	0.0003	0.0002	5-Level	0.0020	0.0053	0.0026	0.0015		0.0023	0.0009
T120I5D	9	90	0.0014	0.0011	0.0003	0.0002	5-Level	0.0020	0.0053	0.0027	0.0017		0.0023	0.0012
T120I20	9	94	0.0015	0.0015	0.0002	0.0002	5-Level	0.0017	0.0047	0.0021	0.0013		0.0023	0.0008
T120I50	9	105	0.0015	0.0014	0.0002	0.0002	5-Level	0.0022	0.0059	0.0024	0.0013		0.0025	0.0008
T120I50D	9	111	0.0014	0.0013	0.0002	0.0002	5-Level	0.0020	0.0059	0.0029	0.0012		0.0030	0.0007
T120I100	9	100	0.0012	0.0015	0.0003	0.0002	5-Level	0.0018	0.0070	0.0027	0.0015		0.0023	0.0009

5 Contribution of volatilization and fungal degradation to removal of diesel, synthetic fuel, and fish biodiesel from contaminated sand[1]

5.1 ABSTRACT

Hydrocarbon degradation by naturally occurring microorganisms in contaminated soil can play a significant role in overall contaminant removal efficiency. This study investigated degradation of conventional diesel, heating diesel fuel, synthetic diesel (Syntroleum), fish biodiesel and a 20% biodiesel blend by naturally present microbial colonies in laboratory microcosms under favorable environmental conditions. The highest degradation was achieved for Syntroleum and fish biodiesel fuels, with over 48% total hydrocarbon mineralization within four weeks. Heating diesel and conventional diesel fuel showed the lowest amount of total hydrocarbon mineralization with 18–23% under optimal conditions (20°C, 300 mg N/kg soil, 2000 mg fuel/kg sand).

When activated carbon was included in experimental laboratory setups to sorb volatiles in order to study their role, microbial respiration was reduced, especially if the activated carbon was exchanged frequently. When activated carbon was not used, average

[1]Horel, A. and S. Schiewer. 2009. Contribution of volatilization and fungal degradation to removal of diesel, synthetic fuel, and fish biodiesel from contaminated sand. Prepared for submission in Environmental Science and Technology.

mineralization after 28 days was 48% for biodiesel and 23% for conventional diesel. The lowest cumulative CO_2 respiration was observed where the activated carbon was replenished the most often with an average 22% degradation for biodiesel and 9% for conventional diesel. Activated carbon reduced the amount of volatile hydrocarbons available for biological degradation. The amount of volatiles recovered from weekly changed activated carbon was negligible for fish biodiesel but reached 4% for both types of diesel and Syntroleum after 4 weeks.

Fungal colonies were observed on the surface of soil microcosms, especially for fish biodiesel. In experiments with little or no soil present, fungi suspended on a grid in the air space above the fuel with little or no soil were able to grow solely with volatile hydrocarbon compounds as a carbon source.

A carbon mass balance was established based on CO_2 evolved, hydrocarbons recovered from soil, hydrocarbons extracted from activated carbon, and estimated biomass growth based on nutrient consumption. The mass balance was 63–99% closed.

5.2 INTRODUCTION

Microorganisms in soils can adapt to changes in the environment such that if a hydrocarbon fuel spill occurs, some are able to use the contaminant as their main carbon source and natural degradation and contaminant removal starts to take place. Natural biodegradation is one of the cheapest contaminant removal options; however it may take a long period of time. The rate of the biodegradation of a specific contaminant depends

on the surrounding environmental conditions such as temperature, soil moisture content, and type of soil etc. Measuring the respiration rate as CO_2 production is a well accepted method for determining microbial activities in aerobic microbial remediation systems.

However, not all of the initially present hydrocarbons are converted to carbon dioxide or remain in the soil. Horel and Schiewer (2009c) found that 13 to 67% of the initial substrate remained unaccounted for after 17 weeks. Therefore one goal of the present study was to investigate the role of volatilization in overall hydrocarbon removal from soil to better close the mass balance. In hydrocarbon degradation experiments, the short–chained alkenes can be highly toxic to many microorganisms; however they can also evaporate fast into the atmosphere (Atlas and Bartha 1998). The most easily degradable elements are the short chained hydrocarbon compounds, which enable fast microbial colony growth and for this reason are an important component of the biodegradation process. Activated carbon is commonly used for volatile organic compound removal from air. Therefore it can be used in laboratory experiments to measure the volatilization of light hydrocarbons. Activated carbon adsorbs not only some of the volatile hydrocarbon compounds but also moisture, both of which might reduce microbial contaminant degradation in laboratory setups. The absorptive potential of the activated carbon depends on the relative humidity of the air it is placed in, co–adsorption of water reduces the adsorption capacity for organic chemicals (Qi, Hay et al. 2000).

In this research five types of fuel were used: diesel, heating diesel, arctic grade Syntroleum, processed fish biodiesel, and a blend of 20% biodiesel with regular diesel. The arctic grade Syntroleum fuel used here was produced from natural gas and processed through the Fischer–Tropsch gas–to–liquid technique by the Syntroleum Corporation. Syntroleum fuel is less toxic than conventional diesel due to its minimal content of aromatics, sulfur, and heavy metals (FTA 2007). Syntroleum can be used in its pure form or as a blend with conventional diesel fuel (FTA 2007). Since Syntroleum use in cold climates is still in the pilot phase, its environmental effects and biodegradability are less known. Initial research (Chapter 2) showed that Syntroleum is more degradable than conventional diesel fuel due to its higher content of straight aliphatics ($C_{8\text{-}28}$), which are typically more easily degradable than branched or cyclic hydrocarbons occurring in conventional diesel fuel.

Around 2004, fish biodiesel processing became of interest in Alaska as an option for lowering diesel fuel dependency by using a cheaply available alternative source. At that time Alaska generated 8 million gallons of fish oil as a byproduct of the Alaskan fish industry per year (Steigers, Seshadri et al. 2004), which increased up to 13 million gallons by 2007 (AEA 2007). In this study, processed fish biodiesel was investigated, where the raw fish oil had been converted to biodiesel through the transesterification process by a commercial biodiesel plant in Hawaii (Schmid, 2008, Pers. Comm.) (Witmer and Schmid 2008). The oxidized biodiesel was rehabilitated, which included removing oxidated compounds. The rehabilitation of the biodiesel consisted of adding 400 ppm by

volume of the antioxidant ethoxyquin and passing the biodiesel through a bleaching column of clay to remove fats and oxidized components (Schmid, 2009, Pers. Comm.). The proposed application of the fish biodiesel is for use as a heating fuel in rural Alaskan communities, mixed with high sulfur diesel fuel. Collection of the raw fish oil is being organized from different parts of Alaska, from where it could be transported in large qualities from 2009 onwards (AEA 2007). It was noted in prior research (Horel and Schiewer 2009a) (Chapter 4) using soil microcosms to study the biodegradability of fish biodiesel, that fungal growth occurred on the soil surface. To further investigate the ability of fungi to degrade volatile hydrocarbons was one of the objectives of this study.

Overall, the present research investigates the following aspects for all five fuel types: degradation by microbes in soil under favorable standard conditions without activated carbon (AC), degradation in the presence of activated carbon, quantification of the amount of fuel volatilization using activated carbon, degradation of volatile compounds by fungal colonies in soil-free setups where bacterial degradation is minimized. With this global approach, the paper aims to quantify the contribution of different mechanisms for overall hydrocarbon removal from soil and establish a comprehensive carbon mass balance.

5.3 MATERIALS AND METHODS

5.3.1 Standard experimental setup with or without activated carbon

The rate of hydrocarbon degradation was studied as a function of time while varying the

fuel type. For the first part of the experiment, 1 kg of soil (sand) was placed in an airtight 2.5 L container. Quantified amounts (2000 mg/kg dry soil) of the chosen contaminant, arctic-grade Syntroleum, conventional diesel (#2), heating diesel, fish biodiesel (B100), or biodiesel blend (B20) were added to the surface of previously uncontaminated soil. Additionally, a small amount of soil with known existing microbial cultures from previous experiments that were already adapted to diesel fuel hydrocarbons was added to provide an inoculum of microbes. Fertilizer of the type 20-20-20 ($N\text{-}P_2O_5\text{-}K_2O$, where the total nitrogen ingredients were: 20% ammoniacal N, 30% nitrate N, and 50% urea nitrogen) was dissolved in 10 ml of water with a final concentration of 30 mg N/ml of solution, and was sprinkled over the soil surface, achieving a nutrient dosage of 300 mg N/kg dry soil. The soil was not mixed after or during these additions to better represent natural conditions after a spill on the soil surface. The samples were incubated at 20°C. Data were collected during a 4 week period.

Three variations of this laboratory setup were used in order to investigate the effect of activated carbon on biodegradation and quantify evaporation of volatiles: 1) NAC: no activated carbon was used. 2) RAC: regular activated carbon change, where activated carbon was placed in the jar's headspace and changed once per week. 3) FAC: frequent activated carbon change where the frequency of changing activated carbon was reduced over time, i.e. daily during the first week, every two days during the second week, twice during the third week and once during the 4^{th} week. This was done because the release of volatiles was expected to decrease over time.

For RAC and FAC, 10 g activated carbon was placed at the top of each jar, to ensure all volatile compounds from the fuels were captured. At the specified time intervals, the activated carbon was replaced, extracted with methanol, and analyzed using GC/MS as described below in order to determine the amount of volatiles adsorbed.

Alternatively, the amount volatilized was determined by monitoring the weight loss of an open jar containing 5000 mg of a specific fuel without soil or activated carbon.

5.3.2 Experimental setup for fungal growth on volatiles

The second part of the experiment investigated fungal survival, growth and respiration under adverse conditions where moisture was low and only volatile compounds in the air were available as a carbon source. Fungal colonies were grown with conditions similar to the previous experiment. The experimental setup involved the same type of 2.5 L airtight jars however without soil. 2000 mg of a specific fuel was placed at the bottom of the jar. Approximately 10 cm above the fuel, a net with an area of 15 cm^2 was suspended to support the growth of fungal colonies, such that only volatile hydrocarbon compounds were available as a carbon source. Two different setups were used, one involved some soil particles collected with fungal inoculum and placed on the grid, generally less than 1 g; for the other no soil was present, fungal inoculum was placed on the grid. This setup enabled collection of mycelium samples for determination of the fungal species without contamination by soil particles and bacteria therein. No water was added during the first four weeks, creating conditions where the air humidity level was low. The total length of

the experiment was 41 days, with 10 ml water addition at the bottom of the jar at day 28 to increase air moisture level to achieve more favorable conditions for further fungal growth. Experiments were carried out in duplicate or triplicate, with average deviations of 4.6% from the mean.

5.3.3 Respiration measurement

Carbon dioxide is the main product generated when a substrate is completely mineralized in aerobic degradation processes. The respiration rate, i.e. the rate of CO_2 production, was measured as an indicator for microbial activity. Typically, the container was opened once daily for re-aeration and to measure the evolved CO_2, which had been captured in 20 ml of 1N NaOH solution (Page, Miller et al. 1982), also to change the activated carbon from headspace. CO_2 measurements were taken by using a method and formula developed by Stotzky (Page, Miller et al. 1982). The carbonate formed was precipitated with a 0.3N $BaCl_2$ solution.

To relate carbon dioxide production quantitatively to the degradation of hydrocarbons, the following stoichiometric equation for CO_2 production was used, assuming a C:H ratio of $CH_{2.12}$ for Syntroleum (Bergin 2004):

$$CH_{2.12} + 1.53\ O_2 \rightarrow CO_2 + 1.06\ H_2O. \qquad \text{eq. 5.1}$$

This stoichiometric equation applies only to mineralization of the fuel. Use of this equation based on the measured amount of CO_2 produced can on the one hand underestimate the amount of fuel degraded if not all substrate is completely mineralized

but used for the production of new biomass. Therefore biomass growth should be estimated. On the other hand, the fuel degradation may be overestimated if some CO_2 is produced from other carbon compounds originally present in the soil. That scenario is unlikely in the present study. The predominant importance of the added fuel compared to other possible substrates was confirmed by the use of controls without fuel addition. A baseline was developed based on uncontaminated soil samples for comparison with the calculated CO_2 production of the degradation experiments.

5.3.4 Modeling

First–order degradation rate constants were determined by using the integral method based on the calculated amount of substrate still available according to respiration data,

$$\ln C_t = \ln C_0 - k\,t \qquad \text{eq. 5.2}$$

where C_t is the remaining contaminant concentration at any given time t; C_0 is the initial contaminant concentration; and k is the rate constant. According to this equation, in a plot of $\ln C_t$ versus time, the data points should fall on a straight line with the slope $-k$ if first-order kinetics apply, that is, if the rate of degradation is proportional to the remaining amount of contaminant at any given time. The rate constant can be obtained from the slope of a regression line. Since previous research (Chapter 3) had shown that the first order model fit well if separate rate constants were determined for two or three phases, that approach was used here. The length of phases was determined by visual inspection of the 1^{st} order plot and the rate constants for each phase were determined by nonlinear

fitting, minimizing the root mean square error RMSE between model prediction and experimental data as described by (Chapter 3).

5.3.5 GC/MS analysis of hydrocarbons in soil

To evaluate the relationship between substrate use and CO_2 production, gas chromatography/mass spectrometry (GC/MS) analysis was performed (Agilent Technologies 6890N Network GC System coupled to a 5873 mass selective detector) and compared to respiration data. For this purpose, the diesel range organics (DRO) determination was conducted using a modified method that was developed based on the AK 102 (ADEC 2002) and EPA 8270 (semi volatile organics by GC/MS) methods (EPA 1996). Recovery data analysis was performed for determination of the actual hydrocarbon recovery value by the gas chromatography method used. For this purpose, a known amount of fuel was added to the soil and extracted immediately. The average highest recovered hydrocarbon amount for each fuel type was taken as a reference value of 100% for the GC/MS data analysis instead of the theoretical value (calculated by using a generalized stoichiometric equation). This was done due to some interference with the solvent during the first few minutes of the GC/MS spectrum. The lower weight volatile compounds, in particular, are not reliably measured, since their peaks occur at a time when the solvent is still eluted. For the volatile organic compound analysis from the activated carbon samples, the EPA 8260C (Volatile Organic Compounds by Gas Chromatography/Mass Spectrometry) method was used. Fish biodiesel blends were prepared on the volume percent based method (ASTM D 6751 – 08, 2008).

5.3.6 Identification of fungal species

Isolates and DNA extraction — twenty four specimens were collected from several experimental setups from the first day up to 4 weeks after of visual fungal growth was observed, with fish biodiesel as substrate. DNA was extracted from small samples of fresh mycelia using the DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA).

PCR and DNA sequencing — The entire internal transcribed spacer (ITS) + 5.8S ribosomal subunit gene region of the nuclear ribosomal DNA (rDNA) repeat was amplified using polymerase chain reaction (PCR) in reaction mixtures containing 1.75µl Ultrapure Water (Invitrogen), 1µl 10x Herculase PCR buffer (Stratagene), 0.05µl 100mM dNTP mixture, 25mM of each dNTP (Applied Biosystems), 0.2µl Herculase DNA polymerase (Stratagene), 2 µl of 1µM forward primer, ITS1F (Gardes and Bruns 1993) and reverse primer, ITS4 (White, Bruns et al. 1990), and 3µl of template DNA. PCR reactions were performed using the following temperature program: 95°C/2 min, 34 cycles of 95°C/0.5 min, 54°C/1 min, 72°C/2 min; and 72°C/10 min. Amplification products were subjected to gel electrophoresis in a 1.5% agarose gel and stained with ethidium bromide for visualization of the bands. PCR products were purified directly using the QIAquick® PCR Purification Kit (QIAGEN, Inc., Valencia, CA). The amplification products were sequenced, with the same primers as in the PCR reactions, using the Applied Biosystems (ABI) BigDye v. 3.1 terminator kit and an ABI 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Bioinformatic analyses — Sequence data obtained for both strands were edited for each isolate using Aligner v. 1.3.4 (CodonCode Corporation, Dedham, MA). Sequences were then subjected to similarity searches using the BLAST algorithm at the NCBI GenBank website to identify samples.

5.3.7 Quantification of bacterial numbers

The soil used in the experiments was not sterilized and thus contained some naturally occurring microbial cultures that survive extreme conditions (–50°C to 30°C). The purpose for using unsterilized soil was to further simulate natural degradation processes. To estimate the number of total hydrocarbon degrading bacterial cells originally present in the soil, the most probable number technique was used.

Triplicate 1g samples of uncontaminated or diesel–contaminated soil that (which was used as inoculum) were suspended in 10 ml 1% wt/vol sodium pyrophosphate ($Na_4P_2O_7$). On 96-well microtiter plates, each well received 20 µl soil suspension, 180 µl of Bushnell-Haas broth growth medium, and 5 µl number 2 diesel fuel (for diesel-contaminated and clean samples) or Syntroleum (for Syntroleum-contaminated and clean samples) as a pure carbon source. The total hydrocarbon-degrader MPN plates were incubated for two weeks at room temperature before being scored for positive growth by using INT dyes to detect presence of respirating bacteria (Haines, Wrenn et al. 1996). Actual values were determined by using a corrected MPN table (de Man 1983).

5.3.8 Soil characteristics

General soil analysis was performed using a Dionex DX 500 ion chromatograph with PeakNet software for nitrite and nitrate analysis and the ASTM standard for sieving. Additional analysis for nitrogen and carbon concentration was done by using an elemental combustion system (Costech Instruments).

The pH of the soil was analyzed before the experiments, using a Mettler Toledo pH meter at 21.8°C. The soil pH value was between 7.07 and 7.25, which is optimal for hydrocarbon degradation (Saadoun and Al-Ghzawi 2005); however fungal colonies have a higher range of acceptance toward more acidic conditions (Leahy and Colwell 1990; Bajpai, Kim et al. 2003), which might be more applicable to field conditions as pristine rainwater has the pH less than 7 (Selker, Keller et al. 1999). The original water content before the start of the experiments was negligible, less than 1%. The bulk density and porosity of the soil was determined by using the ASTM standard. The soil particles were ranging mainly between coarse to very fine sand with less than 0.3% of clayey material. The average bulk density was 1.4–1.6 g/cm^3 for sand. The average porosity of the sand was between 39.7 and 49.3% depending on the amount of clay present in the soil. The average percent of available total nitrogen in the soil was 0.0054% and the fraction of total carbon was 0.165%.

5.4 RESULTS

5.4.1 Effect of activated carbon (AC) use in use in headspace on degradation rates

During microbial degradation of hydrocarbons, commonly four distinctive microbial growth phases can be observed: the lag, exponential, stationary and death phases. During the lag phase, microbes are adjusting to the new carbon source (e.g. contaminant) and degradation rate constants are low. In the exponential phase, microbial growth and contaminant degradation occur at high rates. In the stationary phase microbial numbers reach their peak and plateau. The last microbial growth phase is the death phase, where the microbial numbers and respiration decrease due to a lack of substrate. These growth phases, as they apply to degradation of different fuels in soil microcosms, are further described by (Horel and Schiewer 2009b)(Chapter 3).

Figure 1 shows the logarithm of the remaining substrate concentration (based on respiration data) over time. Initially, substrate degradation was slow, as indicated by a small slope, since the slope corresponds to the mineralization rate constant according to equation (2). The time at which the slope suddenly becomes steeper indicates the end of the lag phase. The length of the lag phase was between 3 and 6 days in experiments with no activated carbon (NAC) or regularly changed activated carbon (RAC) in the headspace (Figure 1, Table 1). These numbers parallel earlier studies where the lag phases in similar conditions were alike (Horel and Schiewer 2009b)(Chapter 3).

However, when frequent activated carbon change (FAC) occurred, a longer time period of up to 20 days (Table 1, Figure 1) was observed, with similar lag phase rate constants as for NAC and RAC. The longer lag phase is likely due to adsorption of easily degradable volatile compounds by activated carbon, which whose degradation was apparently responsible for the earlier transition to the exponential phase for NAC and RAC. For several fuels with FAC, the initial degradation rate constants were higher than in the second phase, resulting in a shift compared to the regular microbial growth phases, e.g. for diesel the rate constants were as follows: $k_{1\text{-}D} = 0.0044\ d^{-1}$, $k_{2\text{-}D} = 0.0016\ d^{-1}$ and $k_{3\text{-}D} = 0.0029\ d^{-1}$ (Table 1). However, even the relatively higher rate constants in the first 11 days had a comparable magnitude to lag phase rates for NAC and RAC, and respiration showed generally a similar trend with lower values than NAC and RAC for the first two weeks. Therefore one could consider several lag phases with different rate constants

Figures 2 and 3 show the daily CO_2 production for the different fuel types with different frequency of activated carbon change. The length of the exponential phase was approximately 10 days for most fuel types (Table 1), with the shortest being 8 days for Syntroleum and the longest 13 days for biodiesel. Trends for NAC and RAC were similar, with a respiration peak occurring at a similar height and time (after about one week) for RAC and NAC in the case of heating diesel, Syntroleum and fish biodiesel, however with a faster decline and generally somewhat lower respiration for RAC. Generally, for all fuel types an increased frequency of activated carbon change noticeably

lowered daily respiration (Figure 3). Apparently, activated carbon removed some volatile organic compounds that otherwise extensively contributed to respiration during the first weeks. For FAC, respiration during the first two weeks was considerably lower, with a lower maximum because lower quantities of easily degradable short chained hydrocarbons were available if the AC was changed frequently. However, after two weeks, when the activated carbon for the FAC setup was changed less frequently (twice in third week, once in 4^{th} week), the daily respiration rate increased for all fuel types (Figure 2b) and exceeded that of RAC by the end of the experiment, reaching similar levels as for NAC (Figure 3). The increase after 2 weeks can be explained by the lower frequency of activated carbon change in later weeks. However this cannot explain the pronounced second maximum on day 26 for fish biodiesel, which was not observed for other fuels. This was likely due additional degradation by fungal colonies which were especially noticeable for fish biodiesel. The most distinguishable differences in respiration over time between the three carbon change regimes occurred for the biodiesel (Figure 1d). As discussed below, fungi, which were able to utilize volatile compounds as their carbon source, largely contributed to degradation of biodiesel and its blends. Consequently removal of volatiles by activated carbon had a large impact on biodiesel degradation by fungi.

Mineralization rate constants were derived from linearized first–order plots based on the remaining contaminant concentration. Figure 1 shows such a linearized first–order plot for determining the overall and two– or three-phase model degradation rate constants for

the three different regimes of activated carbon change for biodiesel and 20% blend. In the case of NAC and RAC, for all fuels except pure biodiesel, the three–phase model applied, because the experimental time period was long enough for the microbes to enter the stationary and/or death phase. For biodiesel with NAC, the exponential phase continued till the end of the experiment, consequently, only a two–phase model was used. For FAC an unusual phase progression was observed, which was caused by changes in the frequency of activated carbon exchange over time as discussed above.

The overall rate constants are summarized in Table 1. On average, overall rate constants increased by a factor of 2 from FAC to RAC and another 1.6 times from RAC to NAC. For diesel a 62% higher overall rate constant was observed for RAC compared to FAC; from RAC to NAC, the rate constant doubled for diesel, which was the largest increase among the fuel types (Table 1). The largest increase in degradation rate constants between RAC and FAC was found for Syntroleum and B100 where the overall rate constants more than doubled (Table 1, Figure 1a).

Figure 2 compares the daily CO_2 production for the different fuel types for two activated carbon change regimes (note the lower y-scale for FAC). It can be seen clearly that biodiesel and Syntroleum had the highest respiration, while the lowest was observed at diesel fuel. For FAC and RAC, late fungal colony growth could be visually observed especially in the case of fish biodiesel. This supports the explanation that for biodiesel, degradation by fungi may have contributed to the high CO_2 production in later weeks

(Figure 2b). For diesel or heating diesel fuel on the other hand, no mycoremediation took place, though the respiration data still follow the same trend of increasing CO_2 production with decreasing frequency of AC change for all fuel types.

5.4.2 Fraction of hydrocarbons mineralized for different fuels and carbon change regimes

Figure 4 shows the percentage of mineralization for different fuel types at the amount at the end of the 28 day experiments with three different regimes for changing the activated carbon in the headspace. Since respiration was very low in uncontaminated blanks between 1.6 and 5.6 mg CO_2/day, mineralization percentages in Figure 4 are based on the assumption that all carbon dioxide released resulted from degradation of the contaminant. For all fuels, mineralization decreased in the order NAC > RAC > FAC. The percentage of mineralization for FAC was only approximately half (or less) of that observed for NAC. This means the addition of activated carbon, especially when done frequently, interferes with mineralization. The main reason for this is likely the removal of easily degradable volatile compounds by adsorption to activated carbon, where they are not available for biodegradation, which reduces CO_2 production. However, additionally, removal of moisture by adsorption to AC may have contributed to lower microbial activity.

The fraction of hydrocarbons mineralized after 4 weeks was the greatest for B100 NAC with 50%. Compared with 37% for regular activated carbon change and 22% for frequent

activated carbon change, this amount is very high (Figure 4). The lowest amount of mineralization was observed in the case of diesel and heating diesel fuels, where less than 10% of cumulative mineralization was achieved with frequent AC change and around 20% without using AC in the experiments.

5.4.3 Nutrient availability

The amount of nutrients in soil influences the effectiveness of overall bioremediation of hydrocarbons. It has been noted by several cold region remediation studies, that nutrient deficient soil inhibits the biodegradation process (Ferguson, Franzmann et al. 2003; Walworth, Woolard et al. 2003; Horel and Schiewer 2009c). The original nutrient amount was adjusted to 300 mg N per kg sand, based on earlier studies investigating similar environmental conditions for microbial degradation such as diesel fuel contaminated subarctic soil (Niemeyer 2003; Horel and Schiewer 2009c). Figure 5 shows the nitrogen depletion from soil at different AC change intervals for the different fuel types. Nitrogen usage correlates with the cumulative percentage of hydrocarbons mineralized in the soil (Figure 4), which means that the more hydrocarbons mineralized, the more extractable nitrogen was depleted from the soil. Interestingly, for biodiesel blend, nitrogen use increased by less than 27% from FAC to RAC, and increased only slightly from RAC to NAC. For the other four fuel types, the increase of nitrogen depletion from FAC to RAC ranged from 116% (heating diesel) to 168% (Syntroleum) (Figure 5). From RAC to NAC nitrogen use increased by 60-120% except for heating diesel.

The amount of nitrogen use can be converted to an estimated production of biomass with the overall stoichiometric formula $C_5H_7O_2N$.

$$\%\ \text{C converted to biomass} = \%\ \text{N used} * \frac{300\ mg\ N_{initial}}{1700\ mg\ C_{initial}} \frac{60\ mg\ C_{biomass}}{14\ mg\ N_{biomass}}$$

According to this equation, for RAC with a nitrogen depletion of 12-23% depending on the fuel type, the contribution to the mass balance would be 9-17% of the initial carbon. For samples with fungal growth, higher nitrogen depletion was observed. Diesel and heating diesel had low estimate biomass production around 10%, while for biodiesel, where fungi were responsible for a significant amount of respiration, 16% of the initially present carbon were converted to biomass according to the above equation. This corresponds to observations of other researchers who noted that bacterial flora assimilates 5–10% of substrate C into new cells, while fungal flora assimilates 30–40% into new mycelium (Sakamoto and Oba 1994).

5.4.4 Volatilization

The carbon mass balance for the total hydrocarbons present in the soil at the start of the experiments in past research was established based on carbon from cumulative CO_2 production plus carbon still present in soil at the end of the experiments plus "other", which could not be accounted for the previous categories (Horel and Schiewer 2009c) (Chapter 2). This unaccounted amount can be due to volatilization, incomplete degradation, or incorporation into new microbial biomass as discussed in the previous section. To investigate the amount that might evaporate from the soil, two types of

volatilization experiments were performed. One was mass based, which involved measuring the change of the mass of a specific fuel in the jar over time (Figure 6), and the other was based on GC/MS analysis of volatile compounds recovered from activated carbon in the headspace of the experimental jar (Figure 7). According to an Energy Laboratories, Inc. report (2005) on conventional diesel, the light carbon compounds, which are more volatile, are approximately between 8% and 11% of total hydrocarbons. In the present study, for diesel and synthetic diesel fuels, mass-based volatilization amounted to 12% and 10% respectively by the end of 28 days, while fish biodiesel showed no or negligible volatilization (Figure 6). Diesel volatilization occured fastest during the first few days; within a week more than 6% of the total hydrocarbon volatilized according to the mass-based methodology. During the fourth week the rate of volatilization decreased with less than 2.5% additional volatilization occurring (Figure 6). Syntroleum on the other hand showed a continuous almost linear decline in mass over time (Figure 6).

Figure 7 shows the gas chromatography results, where biodiesel showed no recovered VOC for weekly activated carbon change. This is consistent with mass-based results from Figure 6. However for frequent activated carbon change (FAC) almost 4% total VOC was recovered for B100, which mainly originated from the second day of AC change. Although mass–based data showed no decrease in the total weight of fish biodiesel, this could be due to the fuel's chemical characteristics. Oxidation can lead to a higher–molecular–weight species through oxidative polymerization (Knothe 2006). Fish biodiesel reacts with oxygen when open to the atmosphere and during the oxidation

process the total mass of the biodiesel could have increased; however volatilization still can occur due to volatile oxidation products (Wadumesthrige, Smith et al. 2008). The largest cumulative VOC recovery occurred in the case of Syntroleum; in the 28 day FAC experiment the amount reached 8.2%. In the case of diesel and heating diesel fuel, the recovered cumulative VOC after four weeks varied between 4.2 and 6.2%, whereby the largest weekly amount of 3.7–3.8% for diesel and heating diesel was recovered during the first week (Figure 7). While values were similar to those from mass-based analysis (Figure 6) for Syntroleum, they were lower for both diesel types. This is not surprising since evaporation can occur more easily in the absence of soil.

5.4.5 Hydrocarbon mass balance for different fuels and experimental durations

In order to determine how much carbon actually remained in the soil as a function of time, different batches of diesel, heating diesel, Syntroleum, pure biodiesel and biodiesel blend degradation experiments were terminated after different time periods for subsequent soil analysis. The first experiment investigated the CO_2 production for 7 days only, the others for 14, 21, and 28 days respectively. Figure 8 shows the carbon mass balance for all 5 fuel types for each week the experiment with regular weekly AC change or frequent AC change (FAC).

In the case of diesel fuel degradation the carbon from CO_2 production doubled from week one to week two (4.1% to 8.2%) and the diesel range organics (DRO) remaining in the soil were reduced by 11.1 percentage points (from 74.2% to 63.1%) (Figure 8a). Smaller

changes occurred during later weeks. Estimated biomass production could account for about half of the "other" carbon in week 4. Heating diesel showed very similar results. The total DRO remaining in the soil for diesel after 4 weeks was 57.1%, which was the highest value among all fuel types.

For pure fish biodiesel, the CO_2 production was already as high as 8.5% during the first week, even though the contamination in the soil remaining was the highest (83.1%) among all fuel types (Figure 8d). At the end of the second week, the cumulative carbon mineralization was three times higher compared with first week and the contamination remaining in the soil already dropped to 56.9. At the end of the fourth week total CO_2 production accounted for 42.6% of carbon and DRO remaining in the soil reached 47.3%. Volatilization accounted for 0–12% of initial carbon. For most fuels, the "other" carbon that was not accounted for by CO_2, DRO in the soil, or volatilization was on average 20%, with lower values for B100 and higher values for B20. Based on nitrogen depletion for RAC, 9-17% of the initial carbon was converted to biomass. Therefore biomass accounts more than half of the "other" carbon. In conclusion, the mass balance could almost be closed. Due to the nature of the experiments which entail some variability, it would be unrealistic to expect perfect recovery.

5.4.6 Degradation of volatile compounds by fungal colonies

To investigate fungal survival solely on volatile compounds, a separate experiment was carried out where fungi were grown on a net suspended above the fuel with little or no

soil present. Fungal growth and respiration was observed in all experimental setups, which indicates that fungal fuel degradation occurred even under non–favorable conditions. During the experimental time frame, the amount of CO_2 production was much lower than in the first set of experiments (Figure 3); however fungal respiration still occurred even after two weeks of non–favorable conditions. Fungal colonies not only can survive on volatile compounds, which can be toxic to most organisms (Atlas and Bartha 1998), but also produced new hyphae and in several cases visual growth could be also observed. . As expected, with some soil particles the daily respiration rate was somewhat higher than without soil, however, the differences are minimal as shown in Figures 9 & 10. Figure 10 shows the cumulative CO_2 production for the different fuel types during the entire experimental duration. Interestingly respiration for biodiesel and its 20% blend was highest, even though biodiesel showed the lowest amount of volatiles (Figures 6 and 7). Apparently these fuels were the best substrates for fungal degradation. Though pure diesel and heating diesel had fairly low cumulative CO_2 production, the overall amount was still substantial, with over 80 mg CO_2 produced by day 28, before water addition, and over 100 mg CO_2 produced by day 41. Most fuels showed fairly similar results varying between 103 – 180 mg CO_2 with some soil particles (Figure 10a) and 85 – 157 mg CO_2 without soil particles (Figure 10b). Unusually high cumulative CO_2 production by day 41 was observed for biodiesel (307 mg CO_2) in the presence of some soil particles and B20 (259 mg CO_2) without soil particles, The sudden increase in CO2 production around day 14 for these fuels was associated with a visible growth spurt of fungal colonies, underscoring the importance of fungi in degradation of biodiesel and its blend.

5.4.7 Enumeration of bacteria and identification of fungal species

Long-term environmental consequences of oil spills in soil are less severe than for coastal ecosystems, mainly due to the presence of fungi and bacteria in the soil (Singh 2006). Most hydrocarbon degraders in soils are bacteria and fungi, which are generally abundant (Leahy and Colwell 1990). In the current study, hydrocarbon degraders as determined by the MPN method averaged $0.94*10^4$/g soil ($0.18–3.63*10^4$/g) for uncontaminated soil samples and $1.57*10^4$ hydrocarbon degraders/g soil for diesel contaminated samples which were used as inocula for all experiments in this study. In soils with low organic matter, the approximate initial aerobic bacteria were found to be between 0.28–$16.73*10^6$/g (Yanagita 1990), which includes non–hydrocarbon degraders. It is therefore not surprising that numbers reported by Yanagita were higher than those determined here. For fish biodiesel samples, visual fungal growth could be observed by the 5^{th} day of the experiment and for Syntroleum by the 6^{th} day. To identify the fungal species, several additional experiments were performed with fish biodiesel contaminated soil without inocula, and samples were taken at different intervals (on first day of visual fungal growth and when a different type of mycelium was observed) from setups with fungal growth (Picture 2). These experiments were carried out for all fuel types; however, only soil with fish biodiesel or biodiesel blends as substrate showed fungal colonization and consequently for DNA sequencing, samples based on other fuel types could not be collected. In earlier experiments, fungal growth had also been observed for Syntroleum contaminated samples, some of which were used in the microscopic analysis (picture 1),

where *Trychoderma* was the main species for Syntroleum. DNA sequencing was performed for all collected fungal samples with the results summarized in Table 2. The fastest fungal growth was observed in fish biodiesel contaminated soils compared with Syntroleum with the main species being *Trichoderma harzinaum* and also *Penicillium* species (Picture 1). In general, all fish biodiesel or biodiesel blend contaminated samples showed fungal growth, which means mycoremediation took place.

Syntroleum contaminated samples showed a high amount of hydrocarbon degrading bacteria averaging $1.05*10^5$ g^{-1} soil often complemented by fungal remediation; although fungal growth could not always be visibly observed. *Trichoderma harzinaum* was also identified in Syntroleum contaminated soil samples in the current study. *Trichoderma harzinaum* is along with *Mortierella* sp. one of the most common soil isolates (Leahy and Colwell 1990).

When diesel or heating diesel was the contaminating fuel type, mainly bacterial degradation occured and visible fungal colonies could not be observed in any of the experimental setups; however other studies concluded that *Penicillium* and *Fusarium* species are petroleum hydrocarbon degraders in cold climates (April, Foght et al. 2000). In other studies, *Trichoderma* sp. showed exceptionally fast growth and *Mortierella* sp. showed limited growth on diesel contaminated medium (Gestel, Mergaert et al. 2003). *Penicillium* and *Aspergillus* species have been frequently reported in literature as filamentous fungi that grow on hydrocarbons (April, Foght et al. 2000; Husaini, Roslan et

al. 2008; Mancera-Lopez, Esparza-Garcia et al. 2008). Husaini et al. (2008) found that the highest average growth rates could be observed in the case of *Trichoderma* species, which would correlate with the current study based on visual observation. During the current experiment *Aspergillus* sp. could not be identified as one of the fungi present in the soil.

5.5 CONCLUSIONS

Activated carbon use in experimental setups influenced degradation for all fuel types. The highest respiration and overall degradation rate constants were observed when activated carbon was not used in the headspace. When the activated carbon was changed more frequently, peaks of daily respiration were lower and the overall degradation rate constants decreased considerably. Activated carbon adsorbs volatile fractions of the hydrocarbons originally present in the contaminated soil samples, also some moisture from the atmosphere. Both are important factors in the microbial mineralization of a contaminant. Short–chained hydrocarbons are important right after the lag phase, when easily degradable compounds are used up the fastest and microbial growth increased exponentially during a short period of time. Moisture in the air and soil also enhances degradation by microorganisms, especially during mycoremediation.

In general, biodegradation was the fastest during the first few weeks of the remediation process. For fish biodiesel, mycoremediation played an important role, whereas microbial remediation without fungal growth occurred for diesel fuel contamination. Fungal

colonies on the soil surface produced a substantial amount of CO_2, which continued to increase for a relatively longer period of time than bacterial respiration and consequently the exponential growth continued much longer.

Volatile compounds were between 3.7 and 8.2% of total recoverable hydrocarbon present in the soil for diesel, heating diesel and Syntroleum fuels based on the gas chromatography data, while mass-based data showed up to 13% during the investigated first 28 days. For fish biodiesel no volatilization was noted in mass-based experiments; however, there were still some recoverable VOC from the GC analysis if activated carbon was changed frequently.

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a) b)

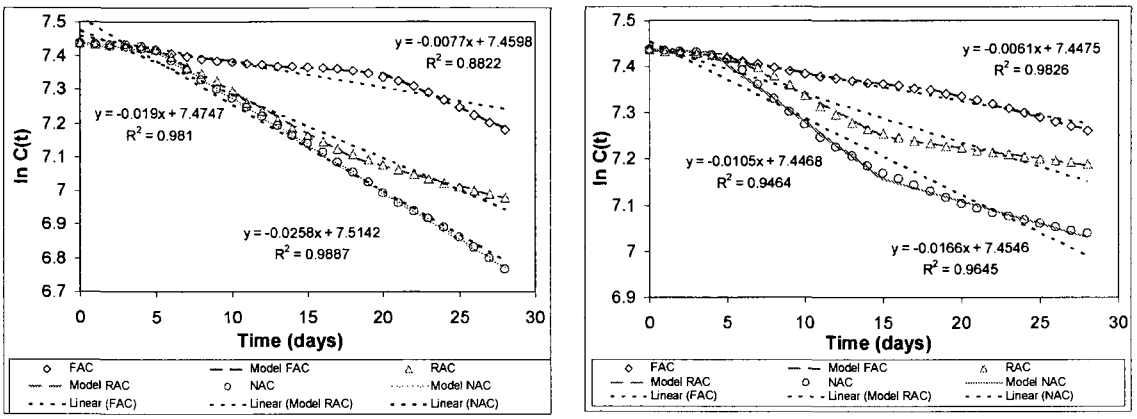


Figure 5.1 Linearized first-order plot for determination of the overall respiration rate constant using the integral method. Conditions: 20°C; 2000 mg/kg fuel; 300 mg N/kg; sand. No activated carbon (NAC), regular weekly activated carbon change (RAC) or more frequent activated carbon change (FAC). Data, overall linear regression, and predictions of 3–phase model, or 2–phase model for biodiesel. Experimental duration: 28 days. a) pure biodiesel (B100) b) 20% biodiesel blend (B20).

a) b)

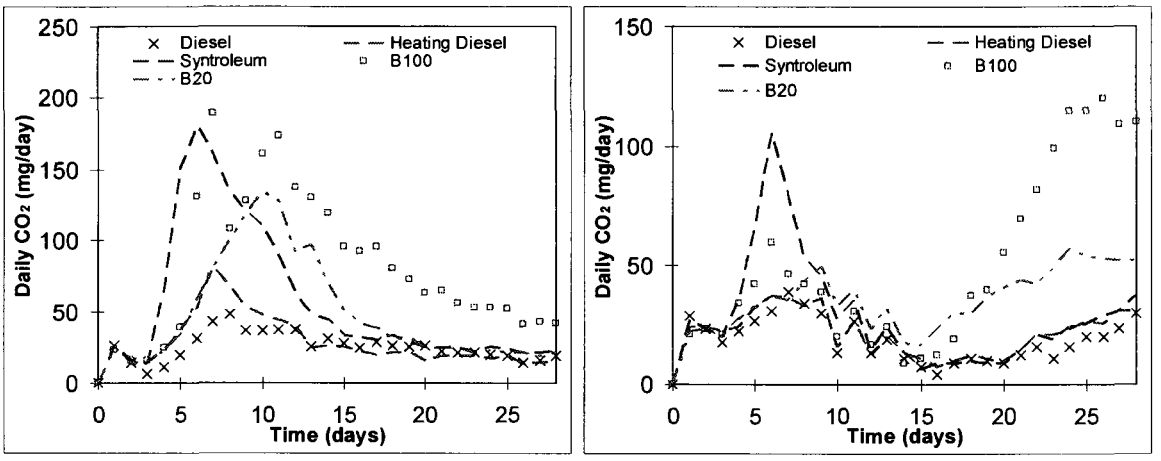


Figure 5.2 Daily CO_2 production for conventional diesel, heating diesel, Syntroleum, biodiesel and biodiesel blend. a) regular weekly activated carbon change (RAC) b) frequent activated carbon change (FAC). Conditions: 20°C; 2000 mg/kg of fuel; 300 mg N/kg; sand.

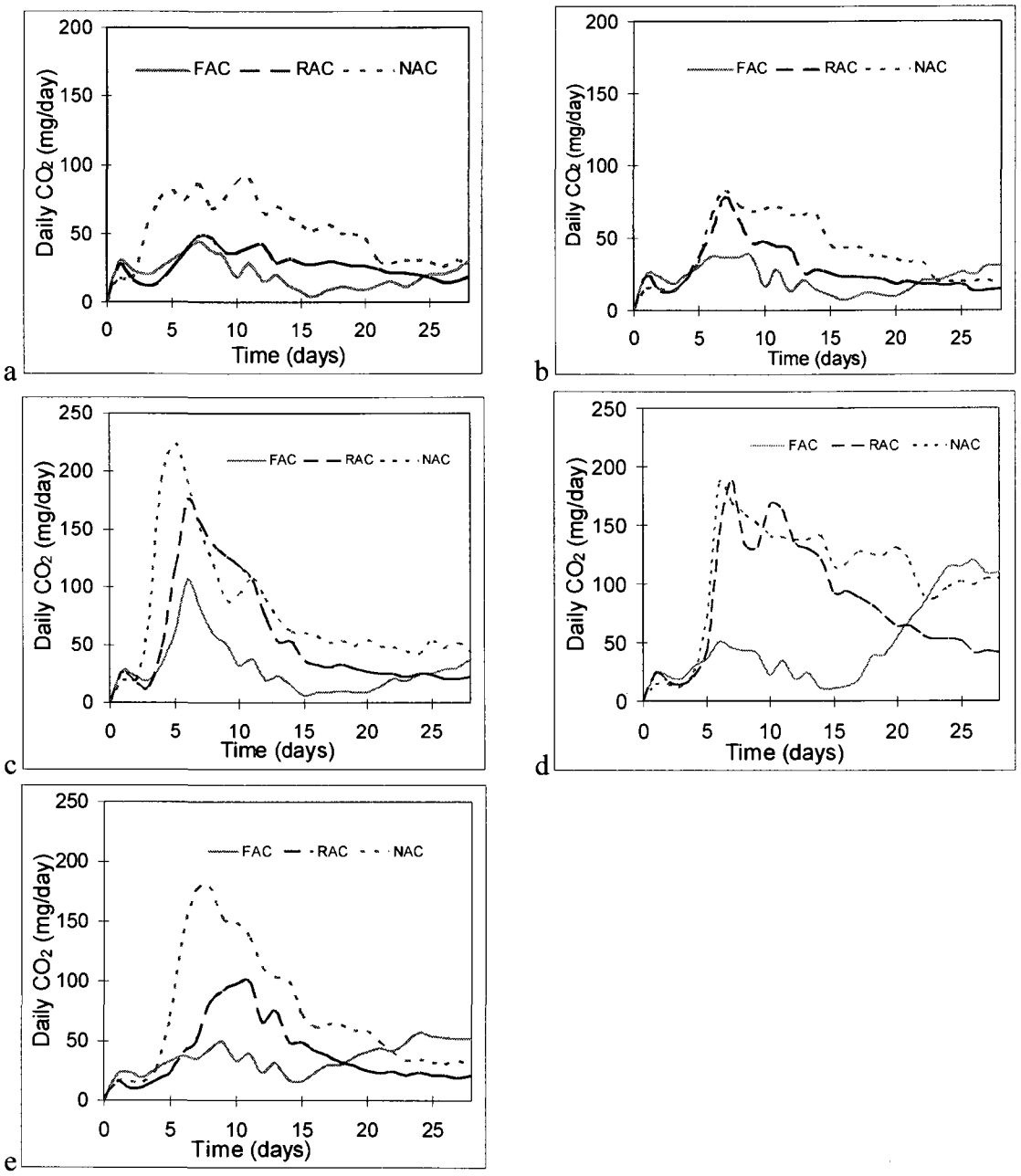


Figure 5.3 Daily CO_2 production for the different fuel types with varying frequency of activated carbon change. NAC no activated carbon change, RAC regular weekly activated carbon change, FAC frequent activated carbon change. Conditions: 20°C; 300 mg N/kg; sand, 2000 mg/kg of fuel a) conventional diesel, b) heating diesel, c) Syntroleum, d) fish biodiesel (B100) e) 20% biodiesel blend (B20).

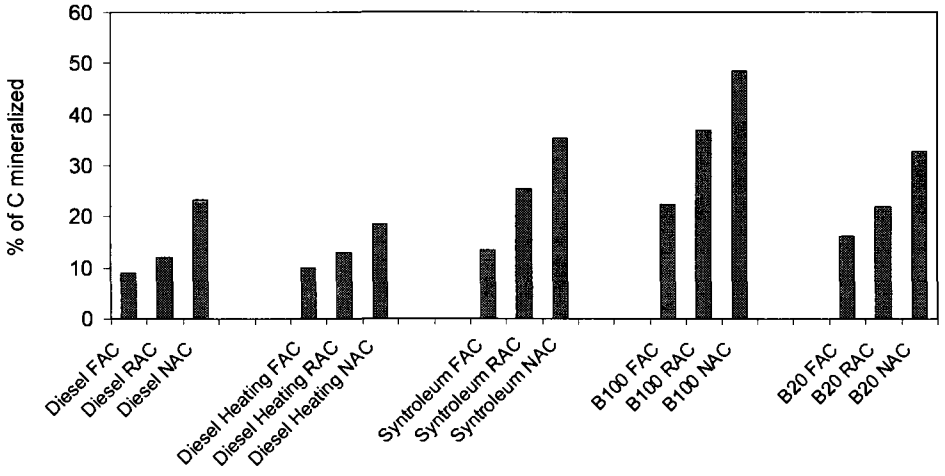


Figure 5.4 Percentage of cumulative mineralization over time for the 5 different fuel types with different frequency of activated carbon change. FAC frequent activated carbon change, RAC regular activated carbon change NAC no activated carbon in the headspace. Conditions: 20°C; 2000 mg/kg of diesel, heating diesel, Syntroleum, biodiesel (B100) and 20% biodiesel blend (B20); 300 mg N/kg, sand. Experimental duration: 28 days.

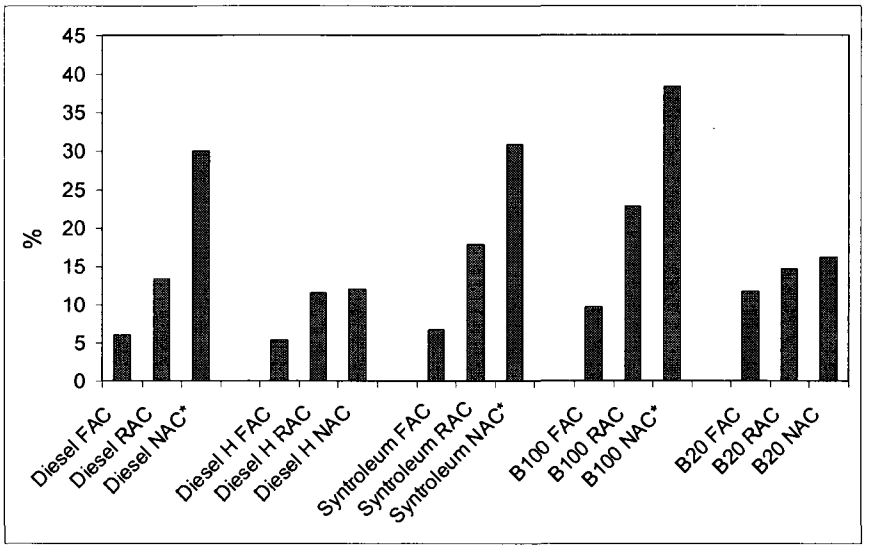


Figure 5.5 Nitrogen depletion from soil at different AC change intervals for the different fuel types. Conditions: 20°C; 300 mg N/kg; sand; 2000 mg/kg of diesel, heating diesel, Syntroleum, B20, and pure fish biodiesel (B100). Experimental duration: 28 days. * HPLC data from different set of experiments

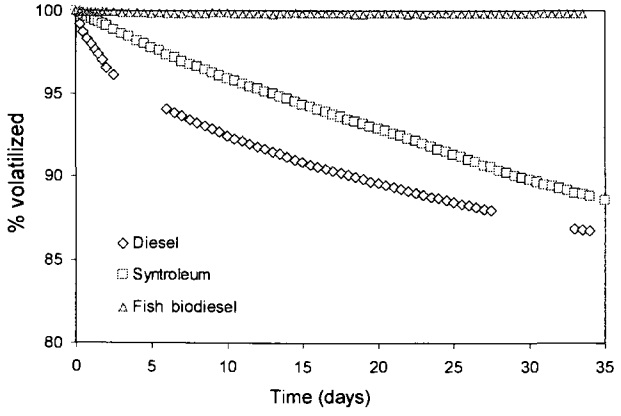


Figure 5.6 Mass based volatilization of diesel, Syntroleum, and fish biodiesel. Data was based on 5000 mg of specific fuel in open jar without soil.

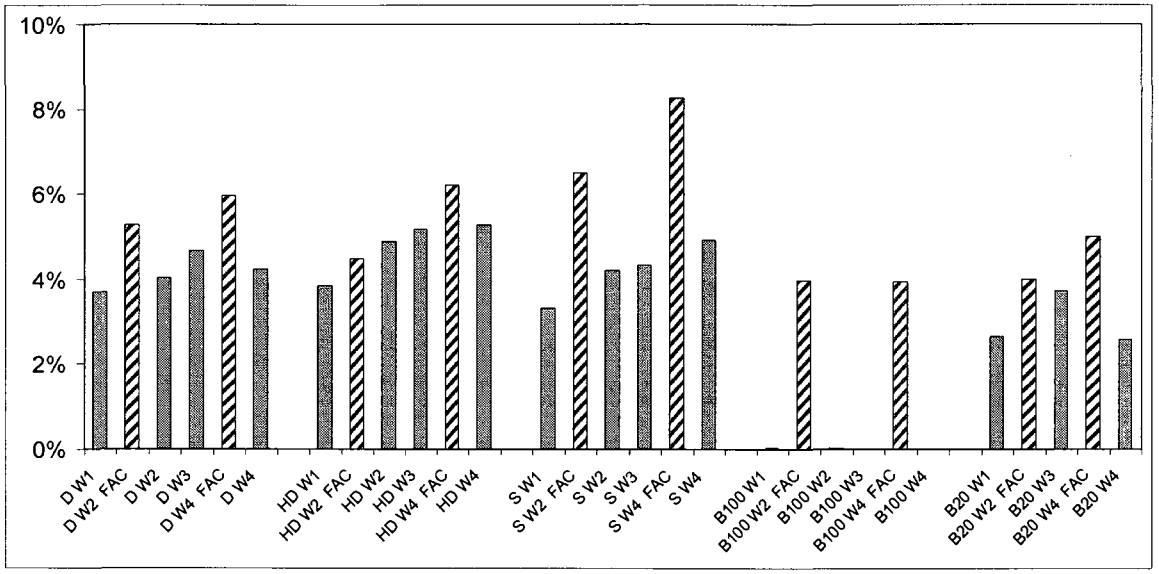


Figure 5.7 Percent of total carbon recovered as volatile organics from activated carbon in headspace for different fuel types over time periods of 1–4 weeks (W1–W4). Activated carbon changed regularly once per week (RAC) except more frequent activated carbon change where marked as FAC. Conditions: 20°C; 300 mg N/kg; sand; 2000 mg/kg of diesel, heating diesel, Syntroleum, pure fish biodiesel (B100) and biodiesel blend (B20).

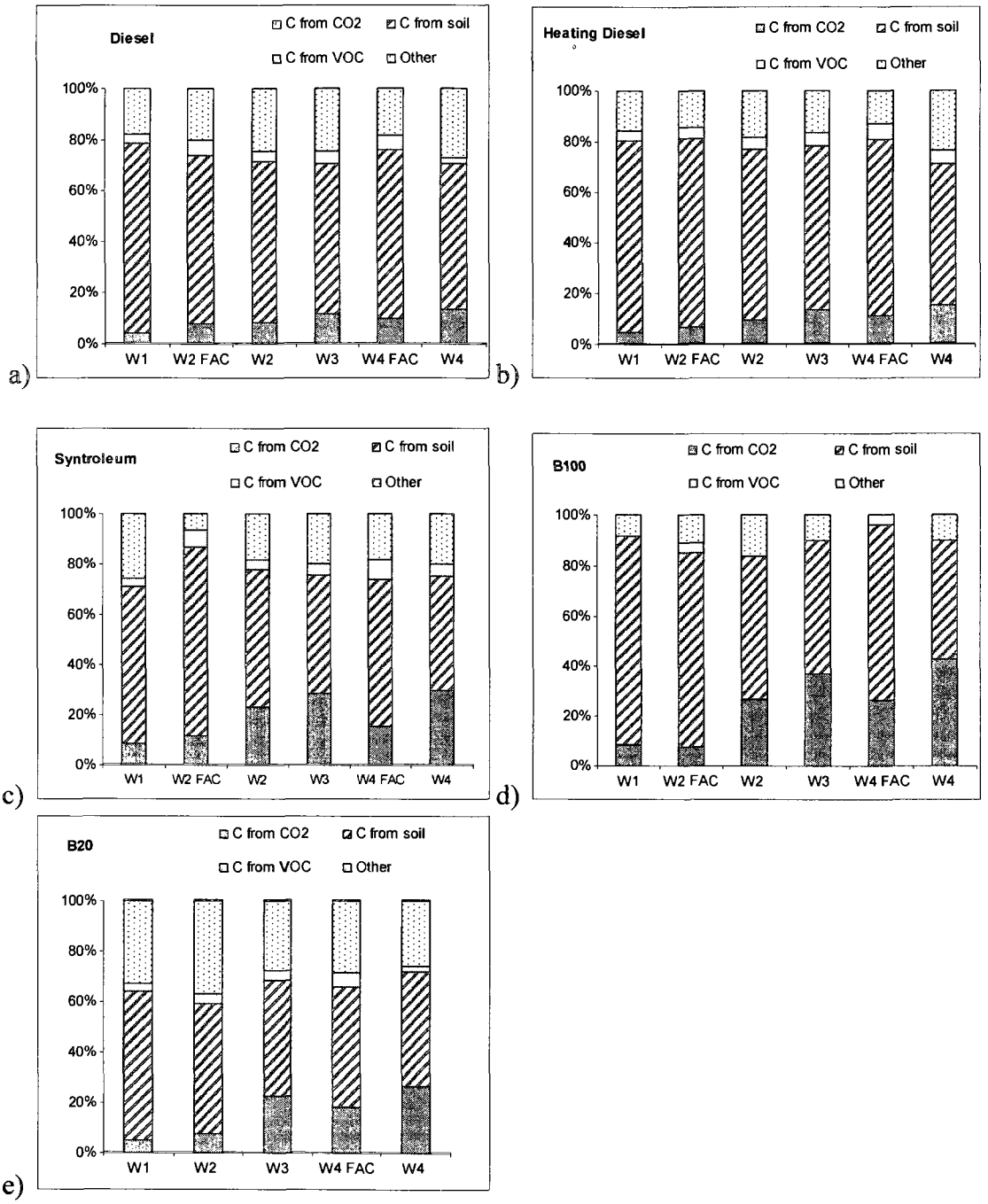


Figure 5.8 Percent carbon mass balance for different fuel types for experiments of 1–4 weeks duration (W1–W4). Activated carbon changed regularly once per week (RAC) except more frequent activated carbon change where marked as FAC. a)

conventional diesel; b) heating diesel fuel; c) Syntroleum; d) fish biodiesel (B100); e) 20% biodiesel blend (B20); 300 mg N/kg sand.

a) b)

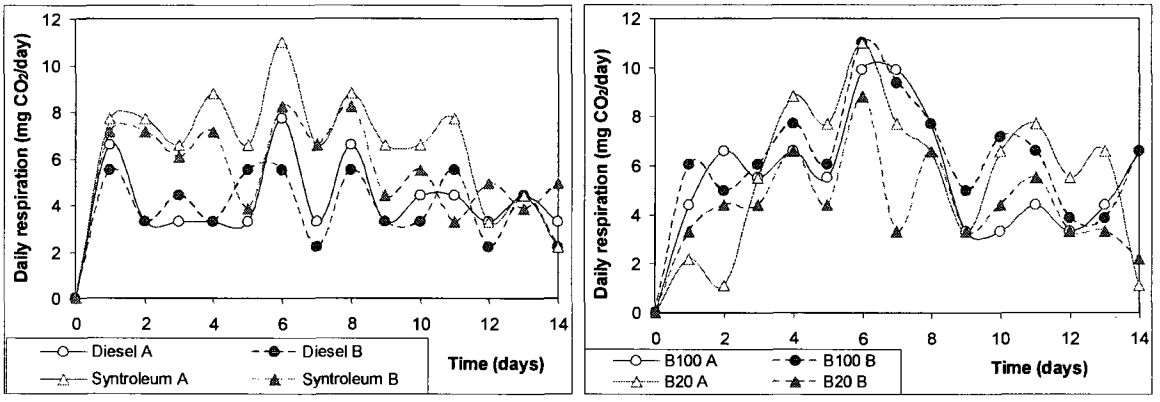


Figure 5.9 Daily fungal CO_2 production based on volatile compounds for different fuel types. Conditions: 20°C; ambient air humidity, 2000 mg/kg of fuel placed without physical contact with soil or fungi. Experimental duration: 14 days. Symbol A with ≤ 1 g of soil, symbol B without any soil. a) conventional diesel and Syntroleum; b) fish biodiesel (B100), and fish biodiesel blend B20.

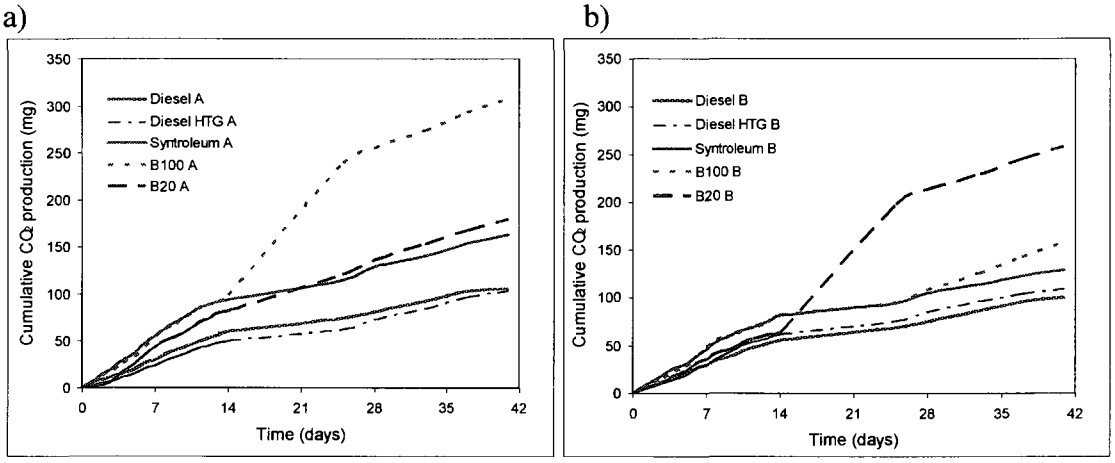


Figure 5.10 Cumulative fungal CO_2 production based on volatile compounds for different fuel types. Conditions: 20°C; 2000 mg/kg of conventional diesel, heating diesel fuel, Syntroleum, fish biodiesel (B100) or 20% fish biodiesel blend (B20) placed without physical contact with soil or fungi. **a)** with ≤ 1 g of soil **b)** without any soil. 10 ml water were added after 28 days at ambient air moisture.

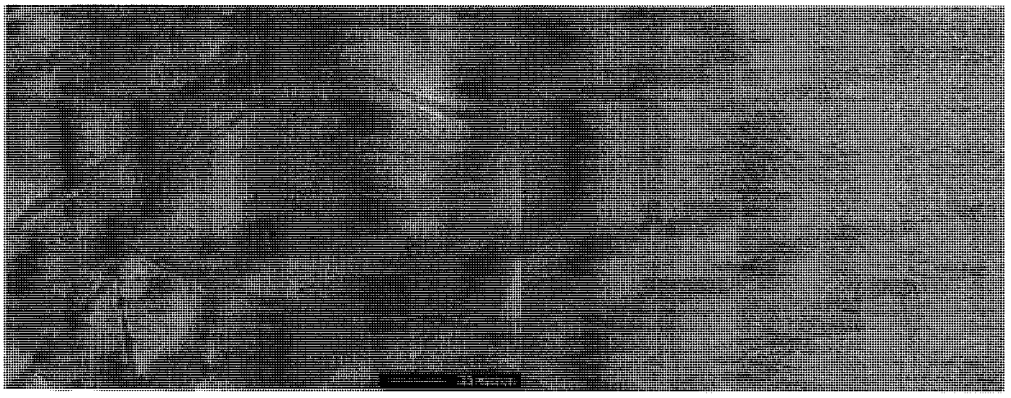


Figure 5.11 Microscopic picture of *Trichoderma* and *Penicillium* sp. Samples were taken from fish biodiesel contaminated soil surface.

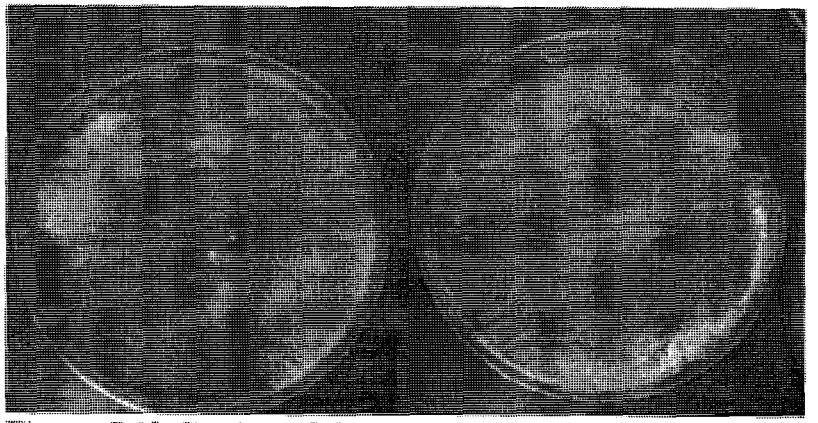
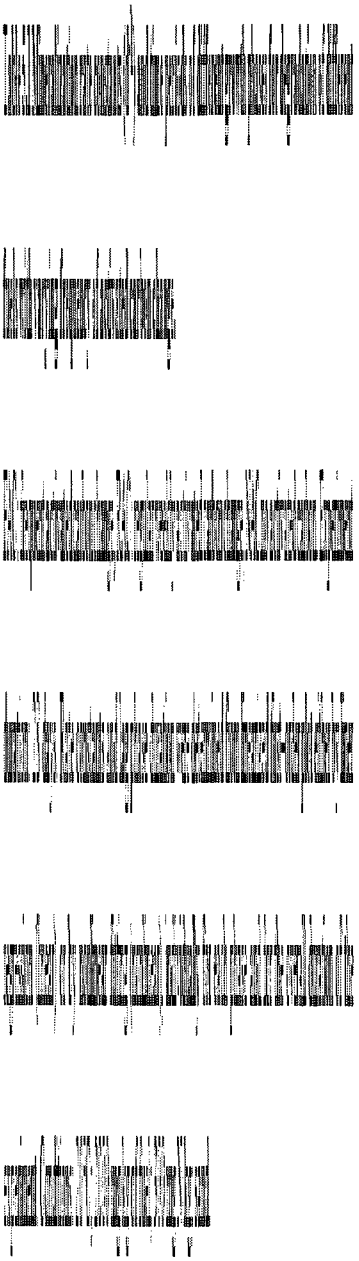


Figure 5.12 Cultivated fungal growth on fish biodiesel medium. Spores were already present in soil samples of same type as in actual experiments. DNA results are shown in Table 2.



Fuel types		Length of lag phase	Phase 1 rate constants (d^{-1}) k_1		Length of exponential phase	Phase 2 rate constants (d^{-1}) k_2		Phase 3 rate constants (d^{-1}) k_3		Overall rate constants (d^{-1})	r^2	RMSE	
			Nonlinear	Linear		Nonlinear	Linear	Nonlinear	Linear			Nonlinear	Linear
Diesel	FAC	11	0.0044	0.0045	12-20	0.0015	0.0016	0.0029	0.0032	0.0029	0.9623	0.0014	0.0015
	RAC	6	0.0031	0.0026	7-16	0.0030	0.0027	0.0035	0.0037	0.0047	0.9897	0.0016	0.0053
	MAC	3	0.0050	0.0045	4-16	0.0123	0.0127	0.0061	0.0062	0.0165	0.9825	0.0025	0.0032
Heating Diesel	FAC	11	0.0047	0.0049	12-20	0.0013	0.0019	0.0040	0.0044	0.0024	0.9739	0.0015	0.0031
	RAC	4	0.0035	0.0029	5-14	0.0053	0.0064	0.0030	0.0033	0.0054	0.9556	0.0026	0.0030
	MAC	5	0.0034	0.0030	6-17	0.0114	0.0112	0.0049	0.0049	0.0094	0.9759	0.0024	0.0044
Synfuel	FAC	11	0.0079	0.0069	12-20	0.0019	0.0018	0.0041	0.0045	0.0047	0.9365	0.0049	0.0107
	RAC	4	0.0062	0.0044	5-12	0.0123	0.0123	0.0053	0.0055	0.0114	0.9110	0.0045	0.0131
	MAC	3	0.0121	0.0107	4-13	0.0238	0.0229	0.0116	0.0115	0.0162	0.9522	0.0058	0.0235
B100	FAC	12	0.0055	0.0050	13-20	0.0032	0.0029	0.0035	0.0191	0.0077	0.9822	0.0037	0.0055
	RAC	5	0.0041	0.0036	6-16	0.0247	0.0247	0.0119	0.0124	0.0191	0.9505	0.0049	0.0054
	MAC	5	0.0044	0.0039	6-	0.0279	0.0278	n/a	n/a	0.0255	0.9807	0.0035	0.0052
B20	FAC	12	0.0052	0.0050	13-19	0.0042	0.0040	0.0036	0.0032	0.0091	0.9826	0.0028	0.0036
	RAC	5	0.0023	0.0023	6-15	0.0177	0.0184	0.0046	0.0049	0.0165	0.9449	0.0035	0.0103
	MAC	4	0.0017	0.0020	5-15	0.0245	0.0253	0.0096	0.0097	0.0166	0.9645	0.0055	0.0094

Table 5.1 DNA results for fungi samples with identified names with specified confidence level.
Samples were taken from several experimental setups at different time intervals.

Species/Fungi name	Division / Phylum	Confidence %
Giberella avenacea. Fusarium lateritium	Ascomycota	93-97
Mortierella hyaline	Zygomycota	96-97
Penicillium species	Ascomycota	93
Trichoderma harzinaum, Hypocrea sp.	Ascomycota	93-94
Trichoderma harzinaum, Hypocrea lixii strain	Ascomycota	98-99
Trichoderma harzinaum, Hypocrea crassa strain	Ascomycota	99
Fusarium oxysporum	Ascomycota	91

6 Investigation of diesel, Syntroleum and fish biodiesel biodegradation in bench scale clayey sand columns and flow modeling by HYDRUS 2D/3D[1]

6.1 ABSTRACT

Hydrocarbon degradation by naturally occurring microorganisms in soil plays a significant role in remediation processes. Small– and larger–scale experiments were carried out to investigate effects of homogeneous and non–homogeneous contaminant distribution on degradation rates. The objectives of this research were (a) to collect data, analyze, and compare the rates of biodegradation of diesel, Syntroleum and pure fish biodiesel in contaminated natural soil from lab column experiments, and (b) to use these degradation rate constants to simulate the fate and transport of the above contaminants in unsaturated porous media. The rate constants of microbial reaction rates for use in model simulations were obtained by investigating the biodegradation rate, measured as CO_2 production, as a function of time for different contaminant types and concentration levels in a small-scale study during a six week time period. The effect of contaminant

[1]Horel, A., S. Schiewer and D. Misra. 2009. Investigation of diesel, Syntroleum and fish biodiesel biodegradation in clayey sand columns in bench-scale studies and flow modeling by HYDRUS 2D/3D. Prepared for submission in Environmental & Engineering Geoscience.

concentration levels on mineralization of hydrocarbons was also investigated. The chemical breakdown of each of the contaminants used in the research was determined by using Gas Chromatography / Mass Spectrometry (GC/MS) analysis. Different types of fuel have similar initial microbial response times. Syntroleum and fish biodiesel had higher degradation rates and microbial respiration compared with diesel fuel. Numerical modeling was used to simulate the transport and fate of the constituents of all investigated fuels in vadose zone conditions similar to lab column experimental setups using HYDRUS 2D/3D. The simulation involved different reaction parameters and stress conditions to understand and distinguish between the fates of the constituents in contaminated soil columns.

6.2 INTRODUCTION

Biodegradation of hydrocarbons in soils plays a significant role in the overall remediation process. In areas where excavation of the contaminated soil is not feasible, in–situ remediation is applied, in which case the time to achieve contaminant removal is crucial. One of the main reasons why fast soil remediation is important is to avoid groundwater contamination, which might affect drinking water sources. Depending on several factors, such as the contaminant type, amount, soil properties and environmental conditions, the time of the contaminant to reach the groundwater varies.

In this paper, the substrate transport and biodegradation of four types of petroleum and non–petroleum–based hydrocarbons were investigated with diesel as a control substance,

arctic grade Syntroleum, and two types of fish biodiesel. The arctic grade Syntroleum fuel was produced from natural gas and processed through the Fischer–Tropsch gas–to–liquid technique by the Syntroleum Corporation. Syntroleum fuel compared with conventional diesel fuel is less toxic due to its minimal content of aromatics, sulfur and heavy metals (FTA 2007). The degradation process in natural environment of Syntroleum has been studied by the current authors (Horel and Schiewer 2009); however its subsurface behavior and transport have not been studied. Alaska generates fish oil as a byproduct from the fish processing that can be converted to biodiesel through a transesterification process (Steigers, Seshadri et al. 2004; AEA 2007). The two fish biodiesel samples were studied included a highly oxidized biodiesel sample (FBD1) that had been degraded by oxidation and oxidized biodiesel that had been rehabilitated (FBD2), which means the oxidative coumpounds were removed (Witmer and Schmid 2008). The rehabilitation of the biodiesel consisted of adding 400 ppm by volume of the antioxidant, ethoxyquin and also an additional process step; the biodiesel was passed through a bleaching column of clay to remove fats and oxidized components (Schmid, 2009, Pers. Comm.). The cold climate performance, biodegradation properties, and behavior in soil of these fuels is unknown and studies as performed here are necessary.

Earlier studies by the current authors were based on small scale experimental setups, involving 1 kg of soil with typically 2000 mg/kg soil of a specific contaminant. Small scale experiments are generally used in laboratory setups for easy degradation rate constant determination under controlled conditions. Large–scale studies are necessary to

investigate the applicability of the findings in conditions where concentrations of contaminants, nutrients, and oxygen vary with the depth below the surface. It is important to know how easily hydrocarbons migrate in the soil. When a hydrocarbon spill occurs in the field, the contaminant concentration is high where the first physical contact between the contaminant and the soil takes place. During natural attenuation process the contaminant becomes less concentrated when no continuous source is present; however the area and volume of the total contaminated soil can significantly increase. The small-scale studies reported here involved different concentration levels to understand the relationship between degradation rate constants and concentration of the substrates. In larger column studies, field conditions were simulated by spilling a quantified amount of fuel on the top of the soil columns to evaluate contaminant transport and degradation as well as related changes in oxygen and nutrient levels with depth.

6.3 MATERIALS AND METHODS

6.3.1 Small scale experiments

The rate of hydrocarbon degradation was studied as a function of time while varying the fuel types. For the first part of the experiment, 1 kg of soil (sand) was placed in an airtight 2.5 L container. Quantified amounts (500, 2000, 5000, 10,000 or 20,000 mg/kg dry soil) of the chosen contaminant, conventional diesel (#2), arctic–grade Syntroleum, or fish biodiesel (FBD1 or FBD2) were added to the surface of previously uncontaminated soil. Additionally, a small amount of soil with known existing microbial cultures from previous experiments that were already adapted to diesel fuel hydrocarbons was added to

provide an inoculum of microbes. Fertilizer of the type 20–20–20 ($N–P_2O_5–K_2O$, where the total nitrogen ingredients were: 20% ammoniacal N, 30% nitrate N, and 50% urea nitrogen) was dissolved in 10 ml of water with a final concentration of 300 mg N/ml of solution, and was sprinkled over the soil surface, achieving nutrient dosages of 300 mg N/kg dry soil. The soil was not mixed after or during these additions to better represent natural conditions after a spill on the soil surface. The samples were incubated at 20°C. For most experiments, some water was added, maintaining a gravimetric moisture content fluctuating between 4 and 6%. Data were collected during a 6 weeks period.

6.3.2 Larger scale column experiments

The column study was performed over a period of 8 weeks. Each column had a length of 75 cm and a width of 25 cm and was equipped 1.27 cm diameter sampling ports at 10 cm intervals (5 sampling ports per column). Preparation of column study involved water addition to achieve 4.5% GWC before the addition of nutrient, which increased total GWC to 4.8%. Nutrient was introduced to the columns from the top without any mixing into the soil. Before the start of the experiment, fuel was introduced from the top of the soil surface without mixing to simulate natural conditions. The contaminant was percolated through the soil column from the top of the soil column. This setup resulted in higher contaminant concentration at the top of the soil columns and decreasing contaminant concentration with increasing soil depths. Soil samples were taken once a week. At the top of the soil columns, a beaker with 60 ml of 1N NaOH solution was placed for respiration measurement (see section 2.3). Soil samples were taken at the same

time the solution with the absorbed CO_2 was changed, at which time also re–aeration of the columns occurred. Each column was filled up to 60 cm height with soil containing an average of 4.8% of original gravimetric water content (GWC) at the beginning of the experiment. At the top of the soil, a 20 g inoculum of diesel contaminated soil with known hydrocarbon degrader microorganisms already present was added. For diesel and Syntroleum contaminated columns, two oxygen sensors (Apogee Instruments) were placed at 5 and 45 cm below the soil surface to investigate oxygen consumption. Additionally each column had a soil moisture and conductivity sensor (Hydroprobe II) placed at 20 cm below soil surface.

6.3.3 Respiration measurements

Measuring the respiration rate as CO_2 production is a well accepted method for determining microbial activities in aerobic microbial remediation systems. Carbon dioxide is the main product generated when a substrate is completely mineralized in aerobic degradation processes. The respiration rate, as the rate of CO_2 production, was measured as an indicator for microbial activity. Typically, the container was opened once daily for re-aeration and to measure the evolved CO_2, which had been captured in 20 ml of 1N NaOH solution for small–scale experiments (Page, Miller et al. 1982) or 60 ml for the column study. During the daily analysis of the small–scale experiment, CO_2 measurements were taken by using a method and formula developed by Stotzky (Page, Miller et al. 1982), which was modified by a multiplicator factor of three for the large-scale studies. The carbonate formed was precipitated with a 0.3N $BaCl_2$ solution.

To relate carbon dioxide production quantitatively to the degradation of hydrocarbons, the following stoichiometric equation for CO_2 production was used, assuming a C:H ratio of $CH_{2.12}$ for Syntroleum (Bergin 2004):

$$CH_{2.12} + 1.53\ O_2 \rightarrow CO_2 + 1.06\ H_2O. \qquad \text{eq. 6.1}$$

This stoichiometric equation applies only to mineralization of the fuel. The amount of CO_2 measured as a result of microbial consumption of hydrocarbons might be different from the actual hydrocarbon decrease in the soil. Use of this equation to estimate the amount of fuel degraded based on the amount of CO_2 produced can lead to over- or underestimations. The amount of fuel degraded may be underestimated based on CO_2 measurements, since not all substrate is completely mineralized, some being incompletely degraded or used for the production of new biomass. On the other hand, the total fuel degraded may be overestimated if some CO_2 is produced from other carbon compounds originally present in the soil. That scenario is unlikely in the present study since soils with low organic content were used. The predominant importance of the added fuel compared to other possible substrates was confirmed by the use of controls without fuel addition.

6.3.4 Biodegradation kinetics model

First-order degradation rates were determined by using the integral method based on the calculated amount of substrate still available according to respiration data,

$$\ln C_t = \ln C_0 - k\,t \qquad \text{eq. 6.2}$$

where C_t is the remaining contaminant concentration at any given time t; C_0 is the initial contaminant concentration; and k is the rate constant. According to this equation, in a plot of ln C_t versus time, the data points should fall on a straight line with the slope $-k$ if first-order kinetics apply, that is, if the rate of degradation is proportional to the remaining amount of contaminant at any given time. The rate constant can be obtained from the slope of a regression line.

6.3.5 Contaminant analysis in soil using GC/MS

To evaluate the relationship between substrate use and CO_2 production, gas chromatography/mass spectrometry (GC/MS) analysis was performed (Agilent Technologies 6890N Network GC System coupled to a 5873 mass selective detector) and compared to respiration data. For this purpose, the diesel range organics (DRO) determination was conducted using a modified method that was developed based on the AK 102 (ADEC 2002) and EPA 8270 (semi volatile organics by GC/MS) methods (EPA 1996). Recovery data analysis was performed for determination of the actual hydrocarbon recovery value by the gas chromatography method used. For this purpose, a known amount of fuel was added to the soil and extracted immediately. The quantity of the average highest recovered hydrocarbon amount for each fuel type was taken as a reference value of 100% for the GC/MS data analysis instead of the theoretical value (calculated by using a generalized stoichiometric equation). This was done due to some interference with the solvent during the first few minutes of the GC/MS spectrum. The

lower weight volatile compounds, in particular, are not reliably measured, since their peaks occur at a time when the solvent is still eluted.

6.3.6 Modeling mass transfer in column

Computer modeling was performed by using the HYDRUS 2D/3D software with parameters retained from laboratory experiments. The 2 dimensional model was executed with x = 12.5 cm and z = 60 cm. The HYDRUS program numerically solves the Richards equation for variably saturated water flow and advection-dispersion equations for solute transport (Simunek, Sejna et al. 2007). The Richards equation is used to obtain the convective velocity of the water, which is then used in advection-dispersion equation to get solute concentration.

The governing Richards equation for variably saturated flow:

$$\frac{\partial\theta}{\partial t}=\frac{\partial}{\partial z}\left[K(\theta)\left(\frac{\partial\psi}{\partial z}+1\right)\right] \qquad \text{eq. 6.3}$$

where θ is the water content, Ψ is the pressure head, z is the elevation above datum, K is the hydraulic conductivity and t is time. Using the Richards equation the change in soil water content can be calculated.

The partition coefficient (K_d) [ml/g] was calculated from the retardation factor (R) (Torres, Orantes et al. 2006):

$$R = 1 + \left(\frac{\rho_b}{\eta} \right) * K_d \qquad \text{eq. 6.4}$$

where R represents the retardation factor, ρ_b [g/cm^3] is the soil bulk density and η [-] is the porosity. The soil hydraulic properties were modeled by the van Genuchten-Mualem model. For the initial condition the highest amount of substrate for the top portion of the soil column was used; 578 mg/cm^2. For solute transport the third-type boundary condition was applied at the top of the soil column model with no flow boundaries at the sides and bottom. The third type boundary condition states that the concentration gradient on the difference between a specified concentration and the solute concentration at the boundaries. The advection–dispersion solute transport equation numerically solves for velocity (eq. 6.5).

Mathematically (Wiedemeier 1999):

$$v_x C - D_x \frac{\partial C}{\partial x} = v_x C_0 \qquad \text{eq. 6.5}$$

where v_x is the velocity across the boundary, D_x is the dispersion coefficient, and x is the distance across the boundary (Wiedemeier 1999). The solute transport equations consider advection-dispersion transport in the liquid phase and diffusion in the gaseous phase. In the HYDRUS model, the advection–dispersion solute transport equation becomes nonlinear when nonlinear adsorption is considered. For the Richards equation, an iterative process used to obtain solutions of the global matrix equation at each time step. During each iteration a system of linearized algebraic equations is derived and solved

using either Gaussian elimination or the conjugate gradient method (Simunek, Sejna et al. 2007).

6.3.7 General soil analysis

General soil analysis was performed using a Dionex DX 500 ion chromatograph with PeakNet software for nitrite and nitrate analysis and the ASTM standard for sieving. Additional analysis for nitrogen and carbon concentration was done by using an elemental combustion system (Costech Instruments).

The pH of the soil was analyzed before the experiments, using a Mettler Toledo pH meter at 21.8°C. The soil pH value was between 7.07 and 7.25, which is optimal for hydrocarbon degradation (Saadoun and Al-Ghzawi 2005); however fungal colonies have higher range of acceptance toward more acidic conditions (Leahy and Colwell 1990; Bajpai,Kim et al. 2003), which might be more applicable to field conditions as pristine rainwater has the pH less than 7 (Selker, Keller et al. 1999). The original water content before the start of the experiments was also negligible, less than 1%. The bulk density and porosity of the soil was determined by using the ASTM standard. The soil particles were ranging mainly between coarse and very fine sand with less than 0.3% of clayey material. The average bulk density was 1.4–1.6 g/cm^3 for sand. The average porosity of the sand was between 39.7 and 49.3% depending on the amount of clay present in the soil. The average percent of available total nitrogen in the soil was 0.0054% and the fraction of total carbon was 0.165%.

6.4 RESULTS

6.4.1 CO_2 production for different contaminant concentrations

During the mineralization of hydrocarbons by microorganisms, the main product is CO_2. Measuring CO_2 production or O_2 consumption can provide an accurate measure of contaminant biodegradation kinetics with low organic content soil types (Korda, Tenente et al. 1997). In the current experimental setups, microorganisms are the sole cause for degradation of the hydrocarbon compounds. If CO_2 production is minimal, that is an indicator of low microbial density, their inability to function, or slow biodegradation of the contaminant due to environmental affects (Alexander 1994). During the current study, fairly optimal environmental conditions were achieved: 20°C, 300 mg N/kg soil and inocula with known hydrocarbon degrader microorganisms were applied. Previous studies showed high degradation of the substrate during these conditions (Horel and Schiewer 2009), while lowering temperature, using nutrient deficient soils, or not applying an inoculum resulted in a considerable decline in respiration rates. The main difference between the setups was the amount of hydrocarbon entering the system or the available O_2 with depth that might be decreasing the overall degradation rate constants.

6.4.1.1 Batch studies

The cumulative CO_2 production is shown in Figure 1 and the responding total hydrocarbon mineralization in Figure 2 for the different fuel types at different contaminant concentration levels. By comparing the different fuel types, it is clearly

shown on Figure 1 that the largest CO_2 production at the end of a 42-day long experiment was achieved in the case of Syntroleum with 7127 mg CO_2 and the lowest was observed for diesel fuel with a cumulative CO_2 production of 5289 mg CO_2, both over a 42 day period. However, when visible fungal growth was observed on fish biodiesel contaminated samples as early as day 5 (up to 10,000 mg fuel/kg soil samples in case of both biodiesel experiments) mycoremediation started to play a significant role in CO_2 production. Large quantities (above 20,000 mg fuel/kg soil) of the biodiesel might prevent sufficient oxygen flow to fungal mycelia or spores and consequently slow fungal colonization on soil surface occurs as it was observed in the current experiments. Figure 2 shows that the percentage of contaminant reduction was the highest for 500 mg of fuel/kg soil (67% for FBD1 and 37% for diesel contaminated samples). When the contaminant concentration increased in the soil, the total hydrocarbon removal percentage decreased, in the case of 20,000 mg fuel/kg soil, diesel achieved only 7.2% and Syntroleum 9.7% mineralization (Figure 2 e). A similar trend could be observed when the degradation rate constants were calculated based on the logarithm of the remaining concentration in the soil. For example diesel contaminant samples had an overall 0.0131 d^{-1} degradation rate constant when only 500 mg fuel/kg soil was added and the degradation rate constant become much lower, 0.0022 d^{-1} when 20,000 mg fuel had been added. The rate constant for the different fuel contaminated samples decreases with increasing contaminant concentration. An earlier experiment investigated the impact of the substrate concentration on the rates by applying Monod kinetics (Chapter 3). According to the Monod model, the rate increases linearly with the substrate

concentration for low substrate concentrations and levels off at a plateau value for high substrate concentrations. For low substrate concentrations (500 – 4000 mg fuel/kg soil) the relationship between substrate concentration and rate was observed as approximately linear; however at higher concentrations (10,000 – 20,000 mg fuel/kg soil) the plateau value was not reached during the investigated 6 weeks time period and the mineralization versus concentration graph remains linear for a long time.

6.4.1.2 Column studies

Figure 3 shows the cumulative and daily respiration data during a 56–day period. From day one a significant CO_2 production was observed for all fuel types with close to 100 mg CO_2 per day. This amount was released for several days, then the respiration increased; however this increase was not as pronounced as in batch–scale experiments. The daily respiration only doubled during the following days and stayed fairly constant around 200 mg/d for the first three weeks for all fuel types except FBD2, where the average daily respiration continued to increase until it leveled off close to 400 mg/d after 4 weeks. This increase could be explained by the fungal growth, which started at day 14 in case of FBD2 and appeared to be responsible for the additional CO_2 production beyond the bacterial respiration for the other fuel types. For FBD1 fungal growth was observed at day 28. Around day 25 a considerable increase in the daily CO_2 production was observed for all fuel types except FBD2, which remained at a constant high level. At day 45 a sudden increase in the CO_2 production was observed. This increase might have occurred due to a larger sampling from the column soil surface for fungal and GC/MS analysis on

day 42, which might have enabled longer and better oxygen distribution in the soil. During sampling the fungal mycelium was cut and oxygen replacement occurred more freely, resulting in accelerated fungal growth and consequently CO_2 production. In general, diesel showed the most stable CO_2 production with the lowest fluctuations in daily respiration. Syntroleum and both fish biodiesel contaminated soil samples showed a similar cumulative CO_2 production by the end of the 56 day experiment (Figure 3a). Interestingly the trends between the biodiesel samples were not as similar as expected due to similar chemical compositions and environmental conditions; however at the end of the experiment the cumulative CO_2 production was similar.

When comparing the data retrieved from the small-scale concentration study to the data from the column experiments, the values for the column respiration data were only 7.6 – 12.2% of what was expected based on small scale experiments. This extremely low amount of microbial respiration could be due to several factors, such as that the exceptionally high concentration at the top of the soil column became too toxic to the microorganisms, oxygen levels were not sufficient enough or became limiting for microbial growth below the surface of the soil, nutrient distribution in the soil was non favorable, or soil moisture content negatively influenced the degradation process. To evaluate the cause of the lower degradation, oxygen, nutrient, and moisture levels were studied as described below.

6.4.2 O_2 depletion from soil at different depths

Figure 4 shows the oxygen depletion from the soil columns during the first four weeks into the experiment. Two oxygen sensors were placed into the soil columns for diesel and Syntroleum contaminated samples, at 5 cm and 35 cm below the surface of the soil. The sensors were calibrated that all of them read 20.7% for ambient air. The first week seemed the most crucial for oxygen depletion, especially in the top portion of the soil columns where a decrease by more than 2 percentage points was observed for Syntroleum contaminated soil, with a somewhat smaller decrease for the diesel sample. The larger oxygen depletion in the upper part of the column, in spite of re-aeration when the columns were opened for CO_2 measurement indicates a microbial activity at the top of the column, which can be explained by larger fuel concentrations (discussed in section 6.3.4). During the first few days of the experiment every day at the same time re-aeration was implemented, which is apparent by spikes in oxygen levels in Figure 4. The magnitude of these increases was approximately a half percent during each re-aeration event; however this increase was not sufficient to restore the original oxygen amount. During the second week the rate of oxygen depletion was similar for both sample ports with less notable decline over the course of the week compared with the first week. By the fourth week the average oxygen level in the soil became fairly stagnant. At deeper depths in the soil columns, the fluctuation in oxygen concentration due to re-aeration was less significant, and overall oxygen depletion was around 1% for diesel and somewhat less than 1% for Syntroleum. When oxygen supplies becomes limited at the top portion of the soil, and re-aeration does not supply enough oxygen to replenish the column, this

might lead to slower microbial growth, and consequently slower degradation of the contaminant as described in section 6.4.1.2.

6.4.3 Impact of soil moisture on degradation

Soil moisture distribution can have an important influence on degradation rates (Davis, Cort et al. 2003). An earlier study by the present authors investigated the effect of different moisture levels on degradation rates in small scale experiments. It was concluded that within 4 – 12% gravimetric water content (GWC) the degradation rates showed minimal differences (Horel and Schiewer 2009) (Chapter 2). During the current study, the initial GWC varied between 4 and 6%. However, in the large columns the continuously monitored overall moisture content showed somewhat higher values of 4.5–11.3% (Figure 5). During the course of the experiment, additional water was not added to the soil columns other than at the beginning of the experiment, and evaporation was minimized due to the closed system setup. Figure 5a shows the moisture content (GWC) changes in the soil during the 8 week long experiment at 20 cm below the soil surface. By week three, the moisture content reached its maximum value. Due to a no flow conditions at the bottom of the experimental columns, higher moisture content in the soil was expected at the very bottom of the soil columns (Figure 5b) with near-saturation conditions expected at the interface between bottom plate and soil. In general, the soil moisture levels throughout the columns stayed fairly optimal during the course of the study, and would not explain the minimal respiration values in the column experiment.

6.4.4 Nutrient distribution throughout soil column

Soil nutrient levels are very important for biodegradation of organic contaminants. When the soil nitrogen content is too low or too high, it can inhibit the degradation processes (Ferguson, Franzmann et al. 2003; Walworth, Woolard et al. 2003). Earlier studies were based on 300 mg N/kg soil, as it was determined to be a sufficient amount to achieve good degradation rates (Schiewer and Niemeyer 2006; Horel and Schiewer 2009) (Chapter 2). During batch scale studies, a fairly homogeneous distribution of the nutrient in the soil can be achieved easily; however, when the larger scale study without mixing was implemented, a homogeneous distribution of the nutrient was not be achieved as seen on Figure 6 for the end of the 56 day experimental period. The top 10 cm portion of the soil contain most of the nutrient amount, with concentrations up to 800 mg/kg, which is already too high for optimal bioremediation (Niemeyer 2003). Significantly lower nutrient concentrations were found at greater depths (Figure 6). At a depth of 30 cm, nutrient levels were already very low. In general, it might be concluded that the nutrient conditions were not favorable for degradation processes, except at depths around 10 cm, and consequently the unfavorable nutrient distribution could have been a cause for lower respiration values.

6.4.5 Hydrocarbon mineralization

Figure 7 shows the carbon mass balance of small–scale experiments for the hydrocarbon mineralization based on the carbon present in the produced CO_2 and carbon remaining in the soil according to gas chromatography data. The highest achieved percentage of

cumulative mineralization was observed in the case of the lowest amount of contaminant in the soil, 500 mg fuel/kg soil. When the 2000 mg fuel/kg soil was investigated the mineralization values were still significant, 48% of the total hydrocarbon removed by microbial activities in fish biodiesel contaminated soils. The lowest was in diesel contaminated soil, where an average of 26% of total hydrocarbons was mineralized during the 42 day experimental period. The amount of unaccounted or missing carbon from the carbon mass balance was also small, around 20% or lower, for both the 500 and 2000 mg/kg contaminant concentration (Figures 7a and b) compared with higher concentrations (Figures 7 c–e). When the contaminant concentration increased in the soil, the overall hydrocarbon removal decreased. At the highest concentration level (20,000 mg/kg soil), the total hydrocarbon removal was less than 10% for all fuel types. It was expected that too large concentrations would result in less complete mineralization than smaller contaminant concentrations. The influence of the substrate concentration on the rates can be described by the Monod kinetics, where the rates are not proportional to the substrate concentration. According to the Monod model, for low substrate concentrations, the rate increases linearly with the substrate concentration and reaches a constant value for high substrate concentrations (Chapter 3).

Comparing the results from the small scale study to the larger column study mineralization (Figure 8), it was observed that the larger setup correlates with the high contaminant concentration from the respiration data and not the overall contaminant concentration throughout the column. This data corresponds with the data retrieved by

the GS/MS analysis of the soil samples taken at different time intervals at different column depths. After a period of 6 weeks, mineralization in the columns was only 5–8 % (Figure 8a), compared to 8–65% in small scale experiments. The highest amount of the total hydrocarbons that was converted to CO_2 by microbial remediation over a one week period was less than 2% of the initial amount of substrate. Figure 9 shows the results for the large column, comparing respiration versus gas chromatography data for diesel and Syntroleum. The carbon removal based on gas chromatograph data is similar but somewhat higher than mineralization based on respiration data. When a spill occurs, there is a large contaminant concentration at the physical contact area of the medium, which can lead to decrease in the percentage of microbial mineralization of the substrates as the parallel setup between small– and larger–scale studies implicated.

6.4.6 HYDRUS 2D/3D modeling

For better interpretation of the laboratory data, several computer simulations were executed using HYDRUS 2D/3D with a 2-dimensional plane with a length of 25 cm and depth of 60 cm. The HYDRUS program is a finite element model. The governing equation for HYDRUS model is the Richards equation (eq. 3) for water flow, which is solved numerically by HYDRUS, and advection-dispersion equation for solute transport. The model uses the Galerkin finite element space weighing scheme, to solve the first-order Richard's differential equation. The advection-dispersion equation is weighted by regular linear basis functions (Simunek, Sejna et al. 2007). The geometric setups for this purpose were made similar to the actual laboratory test geometry, which mainly allows

for vertical dispersivity of the solute. For simplification of the computer modeling some assumptions were applied. The amount of the contaminant concentration in the soil was assumed to be in the dissolved phase due to relatively small amount of hydrocarbon was introduced to the system (average 2000 mg fuel/kg soil). The solute transport was modeled in the dissolved phase by using the third-type boundary condition (eq. 5), with no water flow at the datum. Transient water and solute flow was based on a 1–dimensional transport along the z–axis. There are two main types of boundary conditions (BC): the first type or Dirichlet BC and the third type BC (Simunek, Sejna et al. 2007). According to Batu and van Genuchten (1990), the third-type boundary condition correctly conserves mass in the two–dimensional system and the first–type (Dirichlet) or concentration–type boundary condition corresponds to a situation that the solute flux at the source decreases with time and over time approaches to the solute flux of the third-type boundary condition. This can lead to significant discrepancies in the calculated concentrations, especially near the source boundaries (Batu 1990). Numerical modeling showed that the solute transport was similar to the column experiments, with slow degradation of the contaminant coupled with slow vertical movement since no water was added that would have caused vertical flow. Two types of soil compactions were investigated in the modeling. The first was a general sand column assuming homogeneity throughout the column. The second type was based on larger clay content in the top portion due to saturation from the large amount of water introduced to the column during experimental setup, which resulted in the clay particles accumulating at the top portion in the original column setup. When the two types of soil were modeled, the top portion of

the soil had clayey sand material, while the second portion from 10 cm depth to the bottom plate had the regular, homogeneous sand. Preliminary results showed that with natural flow of the contaminant approximately 34% of the total contaminant concentration remained in the top 10–15 cm of the soil column at the end of the 56–day period while the bottom portion of the soil column stayed at a fairly low concentration level with less than 350 mg fuel/kg soil. According to the simulation during the first week the bottom 20 cm of the soil column did not receive much contaminant at all, mainly due to the minimal water flow. In the laboratory experiment similar results were observed (Figure 10); however the modeling results were showing even less distribution throughout the soil column than the experimental data.

6.5 CONCLUSIONS

The main objective of laboratory testing is to accurately predict what will happen in the actual field under similar conditions. Small scale laboratory experiments are great tools for degradation rate constant determination. However sometimes, like in this experiment, the laboratory results are not readily applicable to the field or larger scale experiments. In modeling, assumptions are necessary for simplification purposes, due to the fact that modeling becomes very complicated when all the parameters that are affecting the outcome are taken into account, which increases the modeling error. When a fuel spill occurs, homogeneous distribution of the contaminant in the soil column is highly unlikely. The same is the case for other chemicals, such as nutrients or oxygen. When designing enhanced biodegradation techniques, these factors are taken into account and

several studies were conducted that consider mass transfer limitations, e.g. slow or time dependent release of nitrogen into soil (Pritchard and Costa 1991) or enhanced aeration by mechanical technologies.

Degradation rate constants determined in the present small scale laboratory study had higher values than in a column system with specific boundary conditions that do not apply for smaller scale studies. Under favorable conditions, with a fuel concentration of 2000 mg/kg, the mineralization rate constant for Syntroleum in small scale experiments was k_S = 0.0136 d^{-1}, while the rate constant in a column with the same average concentration under less homogeneous conditions was k_S = 0.0012 d^{-1}, which is less than the tenth of the previous. In general, mineralization in columns was much lower than expected. The low amount of microbial respiration and consequently hydrocarbon mineralization might have been due to the exceptionally high contaminant concentration at the top of the soil column, which may have been toxic to the microorganisms or due to the unfavorable nutrient distribution in the soil, again, a high concentration at the top of the soil column could have inhibited the degradation process.

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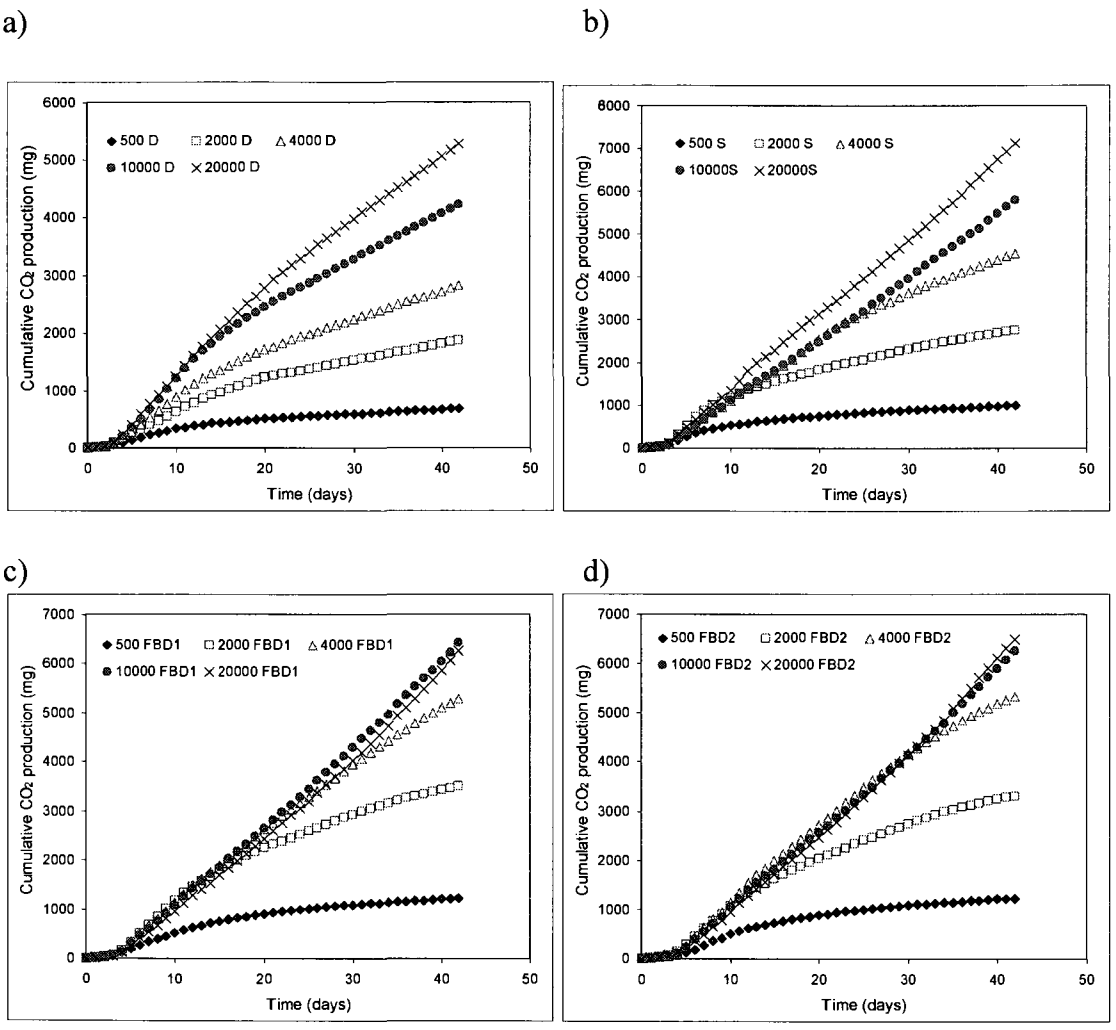


Figure 6.1 Cumulative CO_2 mineralization at different concentration levels based on respiration data. Conditions: 20°C; 300 mg N/kg; sand. Experimental duration: 42 days. **a) diesel; b)Syntroleum; c) FBD1; d) FBD2**

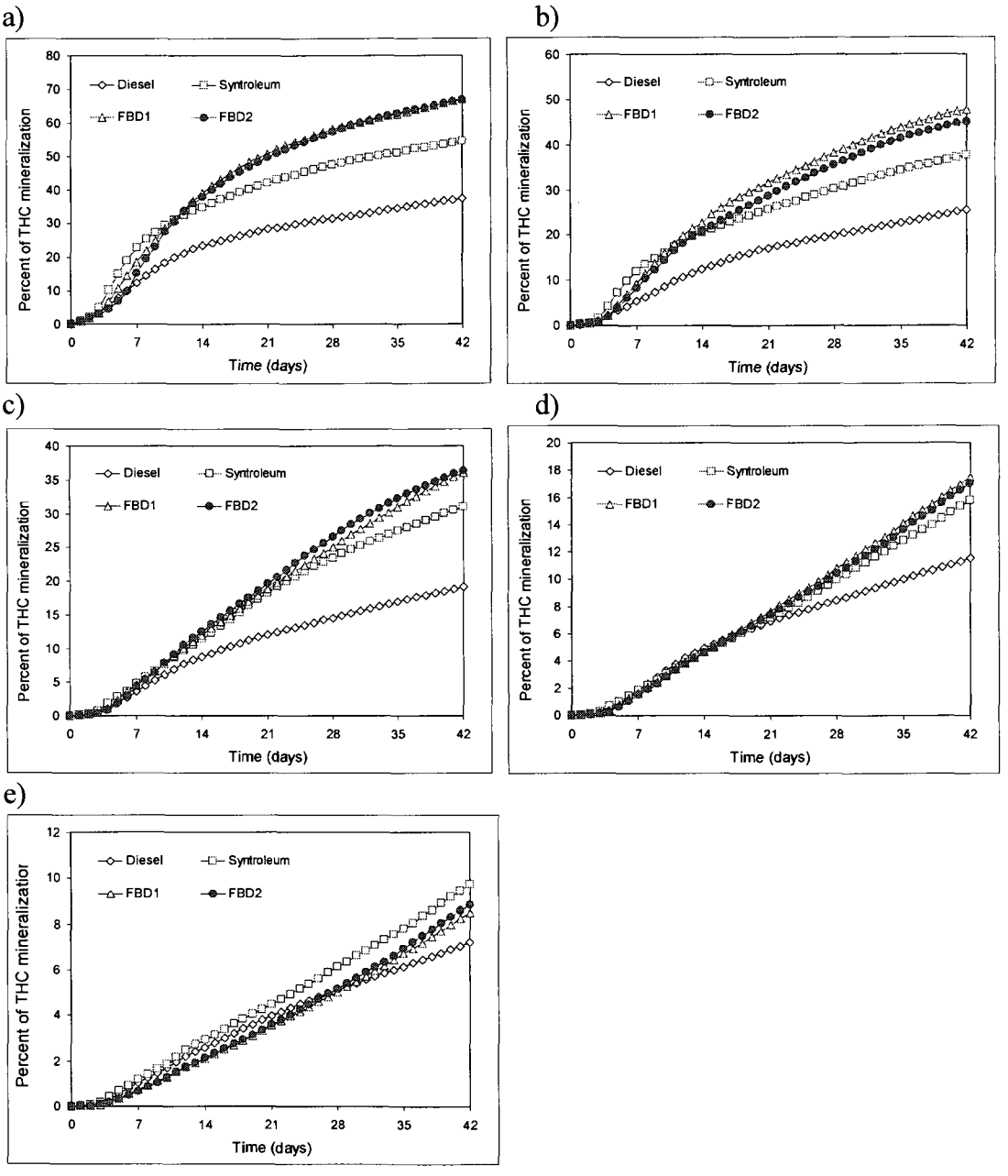


Figure 6.2 Percent of total hydrocarbon mineralized over time for the four different fuel types at different concentration levels. a) 500 mg fuel/kg soil, b) 2000 mg fuel/kg soil, c) 4000 mg fuel/kg soil, d) 10,000 mg fuel/kg soil, and e) 20,000 mg fuel/kg soil. Data based on average respiration data.

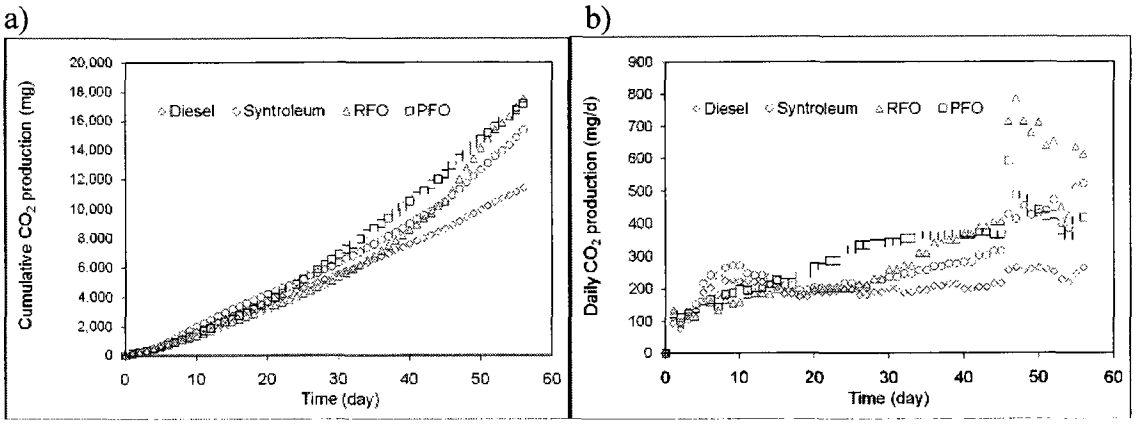


Figure 6.3 a) Cumulative and b) daily CO_2 mineralization for diesel, Syntroleum, FBD1 and FBD2 based on respiration data for the larger scale experiments. Conditions: 20°C, average of 2000 mg/kg of fuel; 300 mg N/kg; sand. Experimental duration: 56 days.

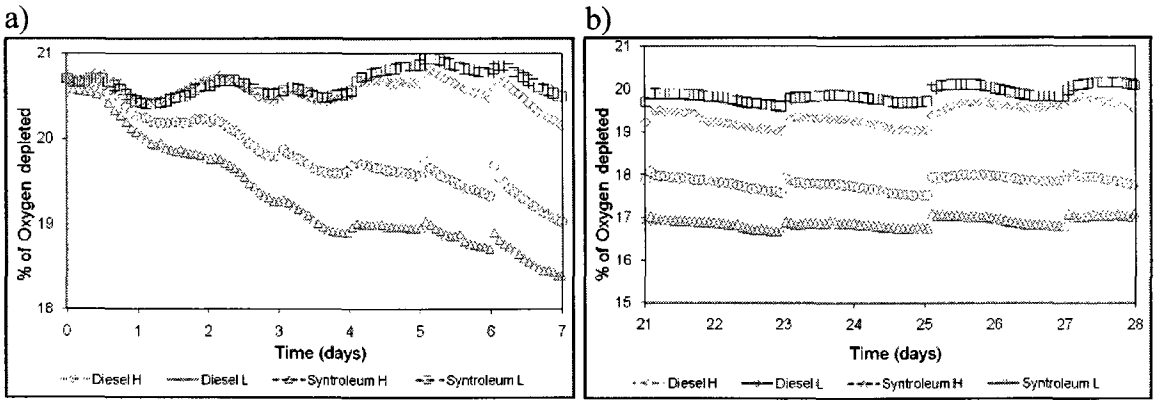


Figure 6.4 Oxygen depletion from the soil at 5 cm below surface (H) and 45 cm below surface (L) during the first and 4th weeks into the experiment for diesel and Syntroleum.

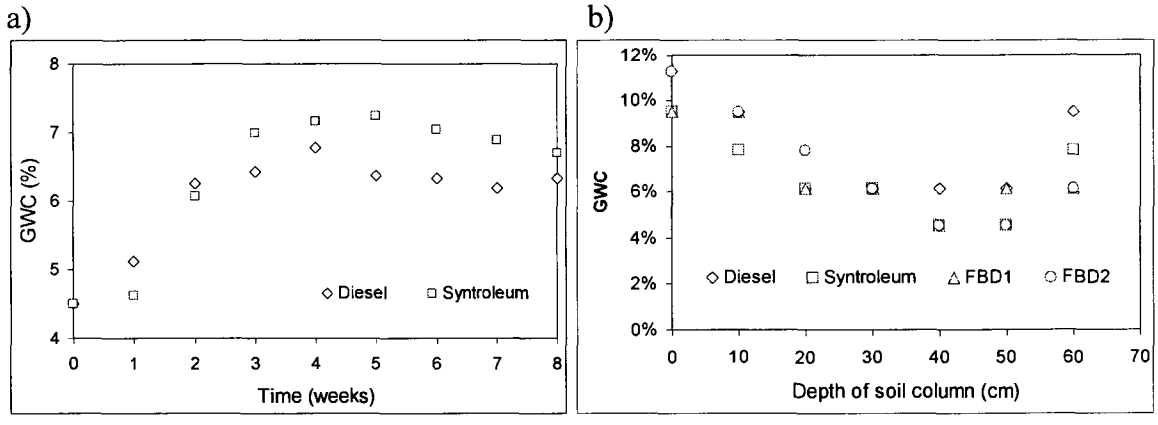


Figure 6.5 Soil moisture content (GWC) distribution in experimental setup a) 20cm below the surface of soil column during the experiment duration measured by moisture sensor and b) at different depths at the end of the 8th week measured by ASTM standard.

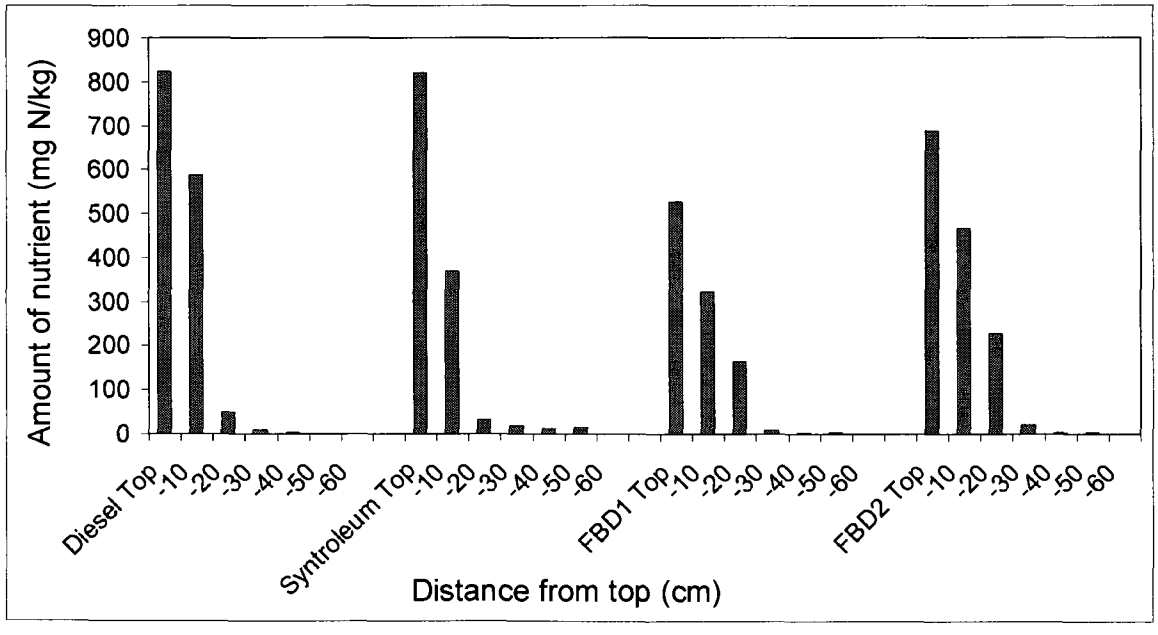


Figure 6.6 Amount of available extractable nutrients (as nitrogen) distribution from soil extractions at different soil depth for Diesel, Syntroleum, FBD1 and FBD2. Conditions: 20°C; average of 2000 mg/kg of specific fuel; 300 mg N/kg; sand.

Experimental data retrieved at day 56 from start of the experiment. Numbers represent average values.

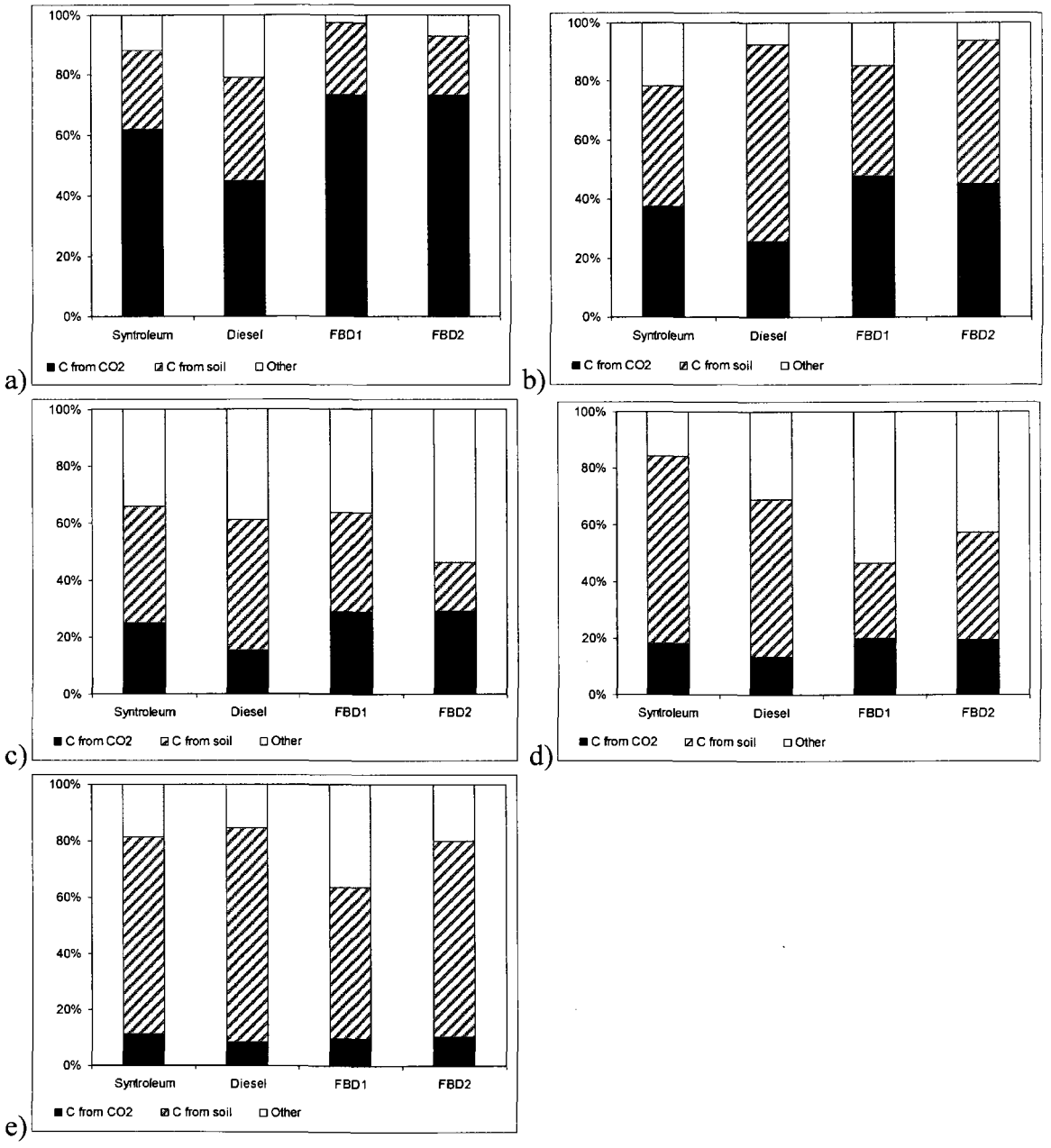


Figure 6.7 Percent carbon mass balance for different fuel types and concentration levels from small-scale experiments. Conditions: 20°C; a) 500 mg/kg, b) 2000 mg/kg, c) 4000 mg/kg, d) 10,000 mg/kg, and e) 20,000 mg/kg per kg soil; diesel, Syntroleum, FBD1 and FBD2; 300 mg N/kg sand. Experimental duration: 42 days.

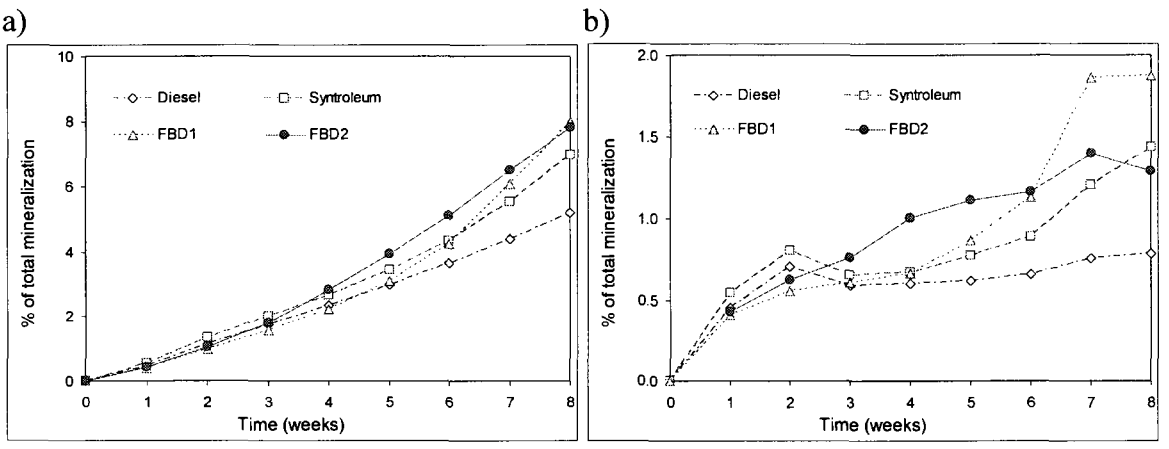


Figure 6.8 Mineralization of Syntroleum, diesel and two types of fish biodiesel over time based on respiration data. a) Percentage of cumulative mineralization. b) Percentage of weekly mineralization. Conditions: 20°C, average of 2000 mg/kg of fuel, 300 mg N/kg.

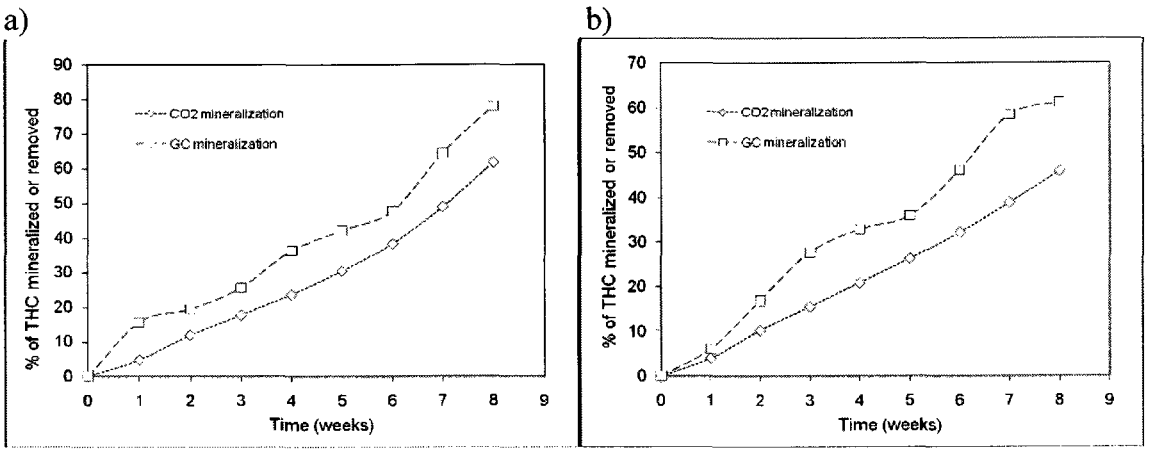


Figure 6.9 Mineralization based on respiration (CO_2) and removal based on GC/MS. Conditions: 20°C, average of 2000 mg/kg of fuel, 300 mg N/kg. a) Syntroleum; b) diesel fuel

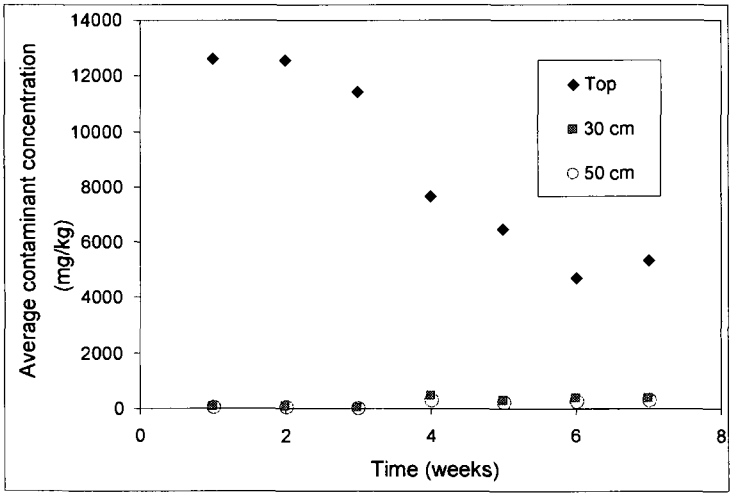
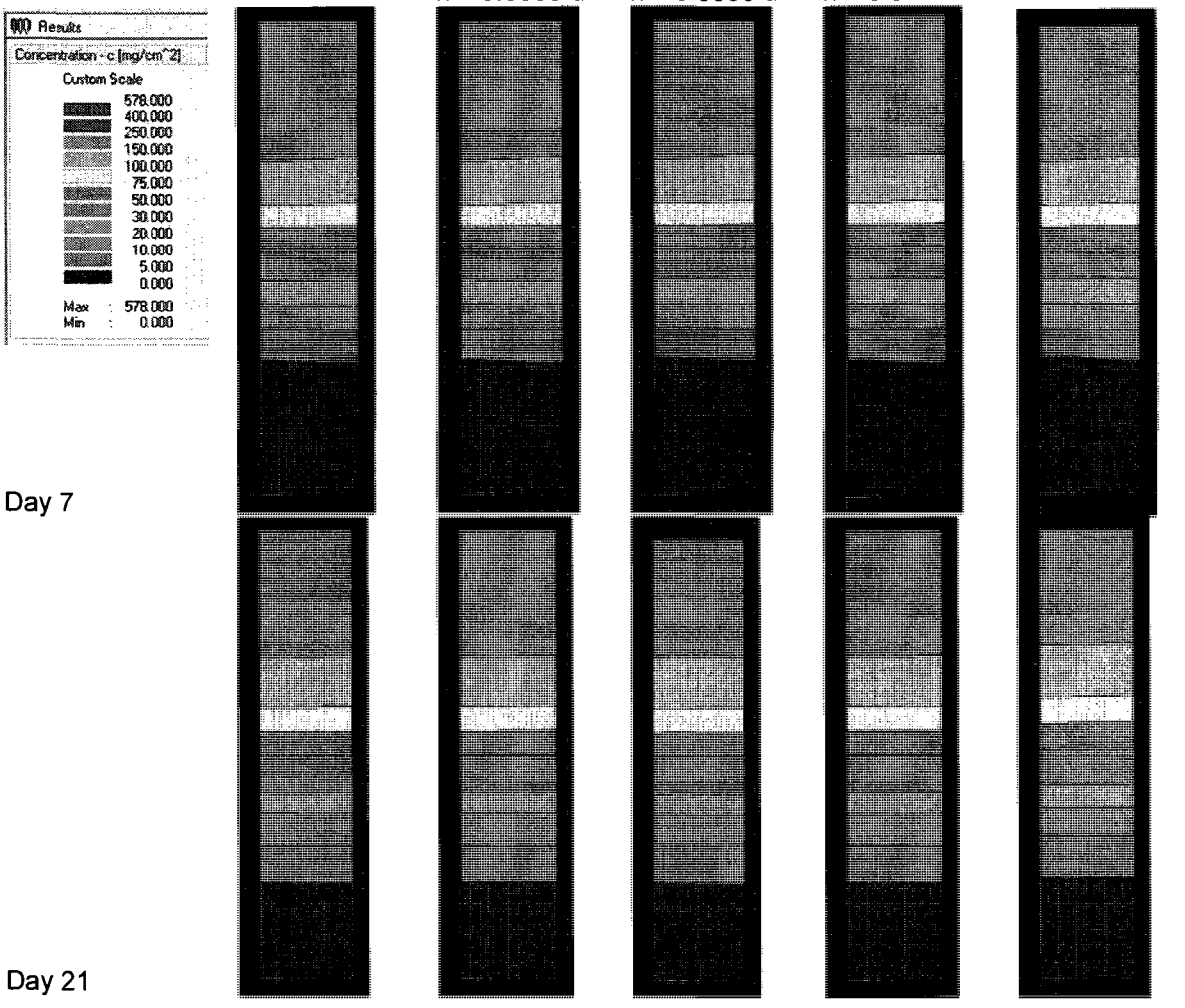


Figure 6.10 Diesel contaminant distribution at different depth of the soil column from GC/MS data.


Fuel type:
Diesel
Syntroleum
Time
Without degradation
Measured degradation rate constants k = 0.0009 d^{-1}
Predicted degradation rate constants k = 0.0086 d^{-1}
Measured degradation rate constants k = 0.0012 d^{-1}
Predicted degradation rate constants k = 0.0136 d^{-1}
Results
Concentration - c [mg/cm^2]
Custom Scale
578.000
400.000
250.000
150.000
100.000
75.000
50.000
30.000
20.000
10.000
5.000
0.000
Max : 578.000
Min : 0.000
Day 7
Day 21

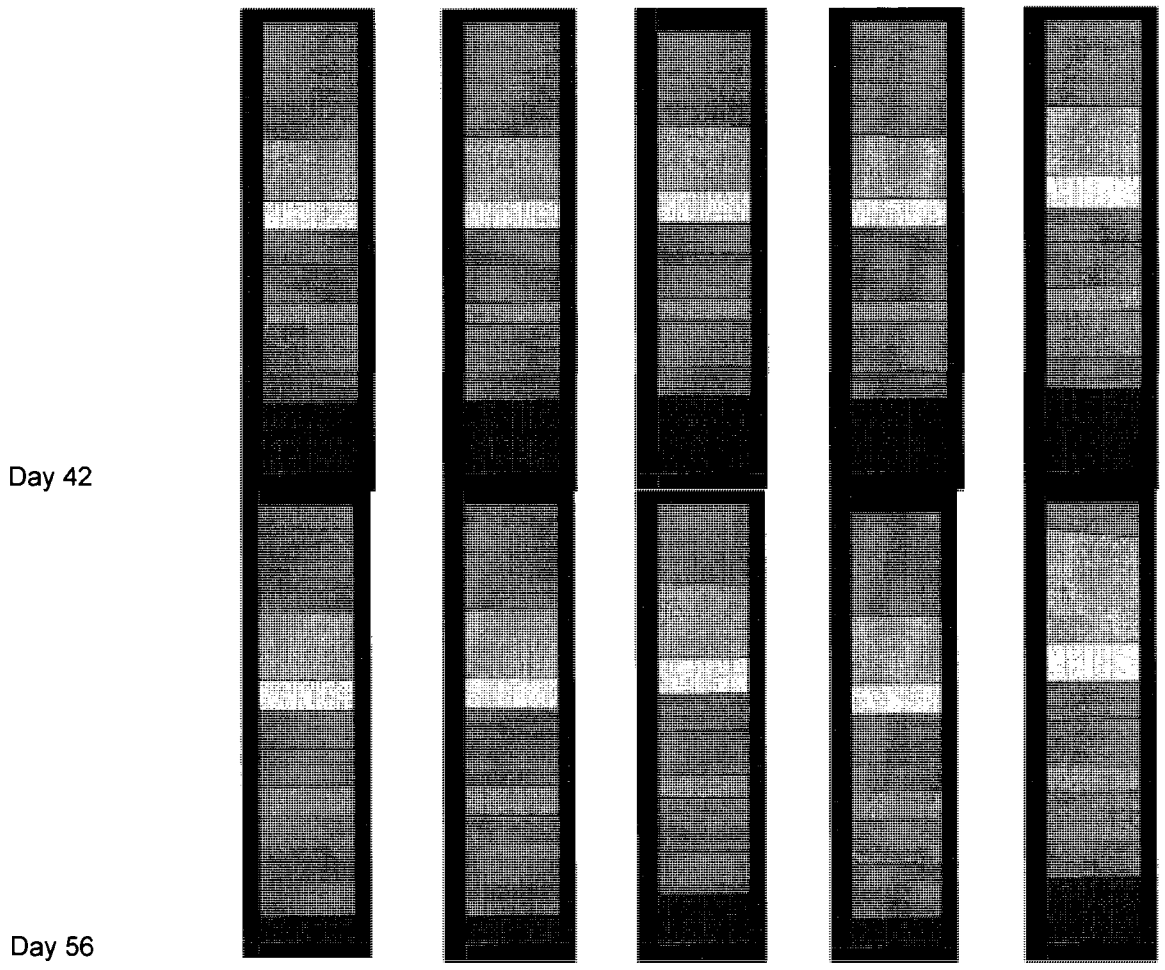


Figure 6.11 Contaminant transport through soil columns. Measured rate constants in the large column experiments, and predicted degradation rate constants were based on small scale studies under optimal conditions.

Table 6.1 Overall degradation rate constants for the four fuel types determined by respiration for the larger-scale experiments. Conditions: 20°C; average of 2000 mg/kg of specific fuel; 300 mg N/kg; sand. Experimental data retrieved at day 56 from start of the experiment.

Fuel types	**Degradation rate constant k (d^{-1})**	**r^2**
Diesel	0.0009	0.9917
Syntroleum	0.0012	0.9803
RFO	0.0014	0.8882
PFO	0.0015	0.9757

7 Overall conclusions

7.1 Degradation of specific fuel types compared with diesel fuel

7.1.1 Syntroleum

A comparison between conventional diesel and Syntroleum fuel showed that average daily and, therefore, cumulative carbon dioxide production was much higher for Syntroleum than for diesel in fertilized soils at different temperatures and contaminant levels. In a four-month experiment at 20°C, a 50% higher cumulative amount of CO_2 was produced for Syntroleum fuel as compared with diesel, and at 6°C the CO_2 amount for Syntroleum was three times that of diesel. Synthetic diesel fuel showed significantly to marginally higher degradation rates in all aspects of this study as compared with conventional diesel fuel. The highest Syntroleum degradation was around 75% total mineralization during four months.

7.1.2 Raw fish oil, fish oil biodiesel and fish oil biodiesel blends

Raw fish oil showed minimal degradation during the first weeks of the experiment, even less cumulative CO_2 production was observed than for conventional diesel fuel within a 4 week long experiment. However, fish oil based biodiesel showed a relatively high mineralization with similar results as for Syntroleum as a substrate. Biodiesel and its blends with heating diesel fuel showed a higher biodegradability than pure diesel fuel. Cumulative mineralization over the experimental duration of 28 days increased greatly when biodiesel was added to the diesel fuel, rising from 18.5% mineralization for diesel

to 23.8% for B5, to 51.6% for B100 at 20°C. Hydrocarbons remaining in the soil and CO_2 produced accounted for 45–85% of the initially present carbon.

7.2 Environmental parameters

7.2.1 Soil types

Regarding the soil types, contaminated sand showed close to 100% higher cumulative CO_2 production after 12 weeks than contaminated gravel under identical conditions. Gravel has little particle specific surface area, limiting its ability to retain contaminants and microbes compared with sand. In sand, adsorption slows the vertical contaminant movement, resulting in more uniform contaminant distribution within the soil matrix, which could entail less toxic effects from the fuel and result in higher mineralization. Additionally, sand has larger porosity, which may have led to better oxygen supply.

7.2.2 Temperature

In general, higher mineralization of the contaminant was observed with increasing temperature. At a temperature of 20°C, microbial activities were highest only a few days into the experiment while at 6°C it took approximately three weeks or longer for the microbes to adapt to the colder environment and peak in respiration rates. While the highest daily CO_2 production was much lower compared with that at high temperature, it took longer before the respiration rates started to decline at the lower temperature. Syntroleum mineralization was 2.5 times greater at the higher temperature, compared with the lower temperature, and diesel mineralization was almost 5 times greater at

higher temperature during a 17 week long experiment. Shorter experiments showed similar results between Syntroleum and fish biodiesel mineralization. Fluctuating temperature between 6°C and 20°C showed fluctuating respiration rates with still significant mineralization at low temperatures.

7.2.3 Nutrients

Two sets of experiments were conducted with nutrient contents of 300 mg N/kg or 0 mg N/kg respectively. Averaging for the whole 17–week study, the overall respiration rate constant without nutrient addition was approximately 36% of the respiration rate constant achieved with addition of fertilizer for Syntroleum. With sufficient nutrient supply for the microorganisms in the soil, the degradation of Syntroleum after 17 weeks was almost 3 times higher at 20°C and 8 times higher at 6°C compared with nutrient–deficient sands. For diesel fuel, fertilizer addition also increased respiration; however, the difference between fertilized and unfertilized samples was not as pronounced as for Syntroleum. The highest nutrient depletion from the soil, which indicated large biomass production, was observed when fish biodiesel samples were investigated.

7.2.4 Moisture

The chosen moisture contents in sand proved to be only a minor factor for gravimetric moisture contents of 2%, 4%, 8% and 12% in Syntroleum-contaminated sand fertilized with 300 mg N/kg and incubated at 20°C.

7.3 Inocula effects on degradation

Addition of inocula that were already adapted to a specific fuel that was present in the soil shortened the lag phase and lead to a higher peak respiration for Syntroleum and fish biodiesel. In general, for the first two weeks, when mineralization rates were highest, biodegradation was faster with previously adapted microbes. However, under favorable conditions significant degradation was achieved with any type of inoculum. For the degradation of Syntroleum and diesel, cumulative CO_2 production after a period of 4 weeks was similar (± 15%) for all three types of inocula. Overall, it could be concluded that while inoculation with adapted microbes initially speeds up degradation, long term results are similar whether or not an adapted microbial consortium was initially present. The amount of hydrocarbons mineralized was highest for Syntroleum as the main substrate and decrease in the order Syntroleum/fish biodiesel > diesel > fish oil, regardless the type of inoculum.

7.4 Microbial growth

The lengths of growth phases were determined based on first order kinetics, which fit the data well when separate rate constants were determined for 2–3 phases. Under favorable conditions, lag phases were less than a week long for all fuel types except fish, and could take as long as 5–6 weeks in sub-optimal conditions. The length of the exponential phase varied between experimental setups; however it was a much longer time period for sub–optimal conditions compared with optimal conditions, where rapid cell growth and

substrate mineralization occurs. After only a short time easily degradable substrate was no longer readily available, thus limiting further degradation.

7.5 Activated carbon use in experimental setups

Activated carbon use in experimental setups influenced degradation for all fuel types. The highest respiration and overall degradation rate constants were observed when activated carbon was not used in the headspace. When the activated carbon was changed more frequently, peaks of daily respiration were lower and the overall degradation rate constants decreased significantly.

7.6 Bacterial versus fungal remediation

For fish biodiesel, mycoremediation played a significant role whereas microbial remediation without fungal growth occurred for diesel fuel contamination. Fungal colonies on the soil surface produced a considerable amount of CO_2, which continued to increase for a relatively longer period of time than bacterial respiration, and consequently the exponential growth continued longer.

7.7 Volatilization

Volatile compounds were between 3.7 and 8.2% of total recoverable hydrocarbon present in the soil for diesel, heating diesel and Syntroleum fuels based on the gas chromatography data, while mass–based data showed up to 13% during the investigated 28 days. For fish biodiesel no volatilization was noted in mass-based experiments.

7.8 Small scale versus larger scale experiments

Degradation rate constants determined in the small scale laboratory studies had higher values than in the column system. Under favorable conditions, with a fuel concentration of 2000 mg/kg, the mineralization rate constant for Syntroleum in small scale experiments was an order of magnitude higher than in a column. In general, mineralization in the larger scale experiments were much lower than expected. The low amount of microbial respiration and consequently hydrocarbon mineralization might have been due to the exceptionally high contaminant and/or nutrient concentration at the top portion of the soil column, which could have inhibited the degradation process.

8 Recommendations for future work

Further investigation of the environmental behavior of alternative fuels in the arctic and sub-arctic environment is recommended. Follow-up work that utilizes and improves the available equipment especially for larger scale studies should be implemented. If it is possible, field experiments should be also carried out.

For future work in the laboratory, the following suggestions are made:

- Optimization of nutrient distribution in soils
- Phytoremediation of non-petroleum based hydrocarbons
- Investigate less grain-sized particles (silt, clay) in the remediation process
- Study the impact of air moisture levels on mycoremediation
- Investigate saturated soil conditions
- Investigate the relationship between activated carbon use as air filter for volatile compounds and biodegradation inhibition in the soil systems

Following are questions future work may seek to answer:

- What are the environmental effects of fuel additives to alternate fuels in cold climate?
- What are the main inhibitors in bacterial versus fungal remediation in a mixed system?
- How can of the limitations in rural areas be overcome?

APPENDIX A

Fungal and bacterial pictures

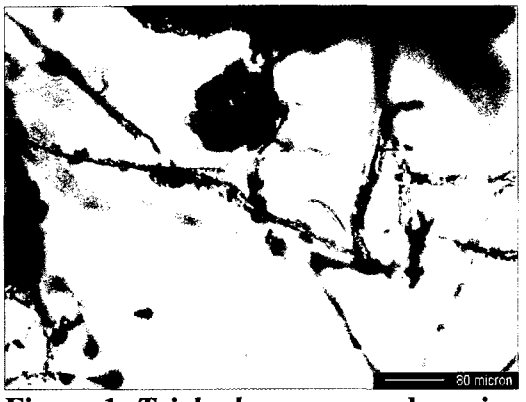


Figure 1. *Trichoderma* sp. under microscope

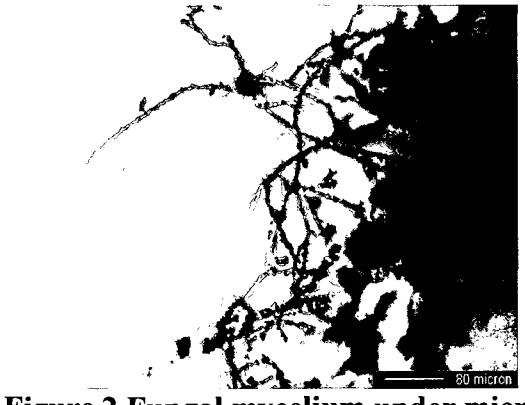


Figure 2 Fungal mycelium under microscope

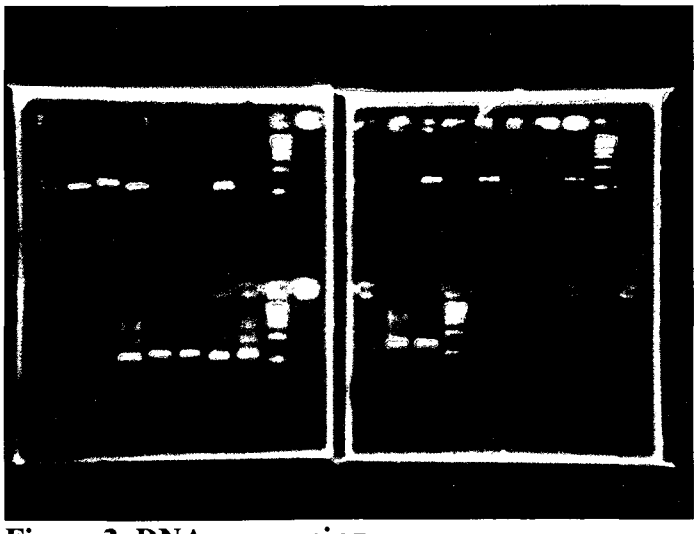


Figure 3. DNA sequencing

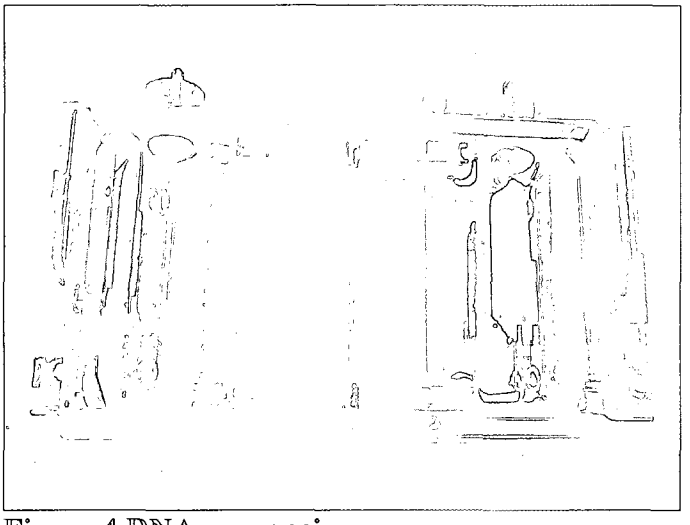


Figure 4 DNA sequencing

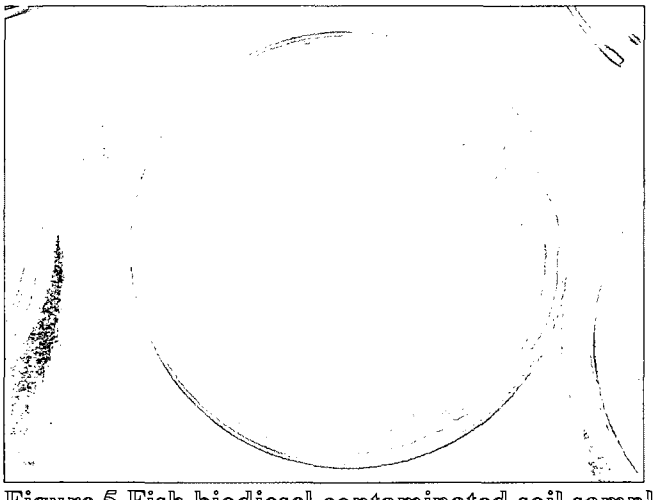


Figure 5 Fish biodiesel contaminated soil samples

Figure 6 Fish biodiesel contaminated soil samples

Figure 7 *Trichoderma* sp. grown on soil surface

Figure 8 MPN procedure – Bacterial count for Syntroleum contaminated soil sample

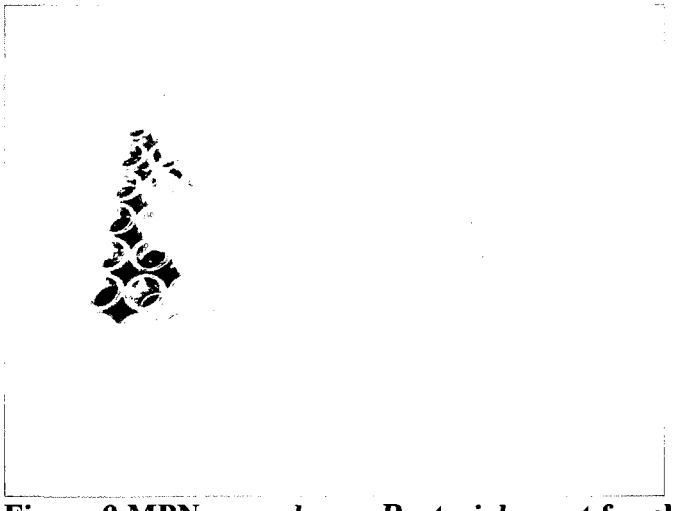


Figure 9 MPN procedure – Bacterial count for clean soil sample with diesel medium

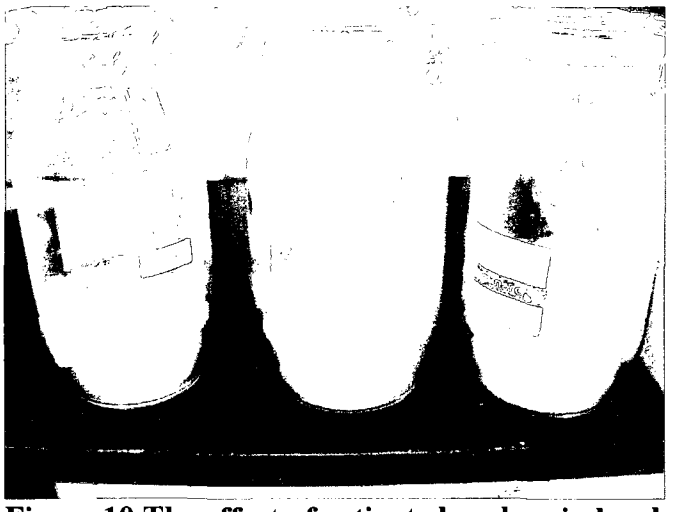


Figure 10 The effect of activated carbon in headspace on fungal growth. Middle jar had RAC while the others FAC

APPENDIX B

Standard deviation

AVERAGE DAILY CO_2 (mg/day)															
		TIME (days)													
FUEL TYPES	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Diesel	0	28.2	18.5	14.7	17.8	26.6	35.8	46.6	43.3	35.4	28.6	35.2	31.0	25.1	23.5
Diesel Heating	0	24.2	16.7	15.4	21.3	34.1	47.9	64.7	52.6	42.9	35.4	37.8	30.8	23.8	22.7
Syntroleum	0	26.4	18.9	15.4	42.9	97.5	152.2	131.5	105.4	96.4	83.8	78.5	53.0	41.1	37.6
B100	0	23.7	17.6	15.2	23.1	40.3	111.3	140.4	97.7	94.6	109.1	111.5	88.2	87.8	76.6
B20	0	22.0	13.6	14.3	21.8	31.0	53.2	67.5	71.5	81.7	81.4	84.4	55.0	65.2	41.3
		TIME (days)													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
Diesel		21.6	19.4	22.7	22.4	20.9	20.5	20.2	18.2	16.0	17.6	20.4	17.1	19.3	24.2
Diesel Heating		20.9	18.0	19.1	19.1	18.3	15.8	18.3	19.8	19.8	20.4	23.7	19.3	22.6	23.1
Syntroleum		26.4	25.3	23.5	25.3	22.4	20.9	21.3	23.1	20.4	24.8	25.9	24.8	25.9	29.7
B100		65.3	66.4	65.3	67.1	61.6	60.9	66.0	68.8	75.9	83.6	84.2	80.3	75.9	75.9
B20		38.1	35.6	34.8	31.2	31.5	30.1	29.7	33.0	34.7	40.2	38.5	36.9	35.2	36.3

STANDARD DEVIATION															
		TIME (days)													
FUEL TYPES	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Diesel	0	1.1	4.1	5.1	5.9	6.5	7.2	10.4	5.9	4.0	10.2	6.9	14.6	5.1	10.8
Diesel Heating	0	1.0	5.7	3.6	3.4	3.9	8.1	21.7	15.3	9.7	16.9	9.0	15.7	2.9	7.8
Syntroleum	0	1.7	3.1	3.4	23.3	43.0	38.6	38.4	42.2	41.8	48.0	38.8	33.1	16.9	21.6
B100	0	1.7	3.0	3.3	6.3	6.4	48.5	73.5	54.0	49.3	80.0	70.6	64.0	58.1	60.4
B20	0	2.6	6.0	4.8	5.9	3.0	16.9	31.4	33.7	41.1	57.2	53.7	37.0	38.4	28.1
		TIME (days)													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
Diesel		12.1	13.3	12.1	10.0	9.6	10.2	7.3	3.9	7.0	3.1	0.8	3.9	5.4	7.8
Diesel Heating		8.6	9.0	8.8	6.3	6.4	6.1	3.9	1.6	1.6	3.9	5.4	8.6	11.7	10.9
Syntroleum		17.5	14.4	12.7	13.3	11.8	10.6	6.1	3.1	2.3	0.8	0.8	5.4	7.0	10.9
B100		47.1	47.0	41.0	25.8	19.1	5.2	2.9	17.9	32.7	43.6	44.3	56.0	46.7	48.2
B20		18.8	10.8	4.6	1.7	5.0	9.2	12.5	12.4	19.4	24.1	23.3	22.6	23.3	21.8